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Abstract from the Scandinavian Physiological Society Meeting *in Goteborg 25–26 April 1980*

Communications

Relationship between local changes in cortical blood flow and extracellular K+ during spreading depression*

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HANSEN A. J QUISTORFF B & GJEDDE, A Relationship between local changes in cortical blood flow and extracellular K during spreading depression. Acta Physiol Scand 1980 109 1–6. Received 19 June 1979 ISSN 0001-6772. Institutes of Medical Physiology Dept. A. and Biochemistry Dept. A. University of Copenhagen Denmark.

Change of local cerebral blood flow in response to a single cortical spreading depression of Leão was studied in the brain During spreading depression, potassium briefly accumulates in the brain extracellular space The cortical blood flow was normal during the maximal rate of increase of the extracellular potassium concentration. The blood flow doubled during the subsequent period of normalization of potassium and remained high for one minute thereafter Thus, potassium cannot be the immediate mediator of the blood flow increase.

Key word Spreading depression, cerebral blood flow brain extracellular potassium concentration, metabolic autoregulation of blood flow

Leão s cortical spreading depression (CSD) of EEG (Leão 1944) is generalized response of the cerebral cortex of animals and man to ariety of noxic nfluences, chemical electrical, and mechanical stimuli (Bureš et al 1974 Šramka et al 1977)

The CSD is accompanied by localized changes of the extracellular concentrations of potassium sodium chloride and calcium (Hansen 1979 Kraig & Nicholson 1978 Nicholson & Kraig 1975 Nicholson et al. 1977 Vyskočil et al. 1972) The perturbation moves from the site of elicitation at a speed of approximately 3 mm/min (Bureš et al. 1974)

The response of the cerebral circulation to the passage of spreading depression was studied by Leão (1944b) Burešova (1957) Tschirgi et al 1957) and Van Harreveld & Ochs (1957) all of whom observed dilatation of pial vessels preceeded by moderat constriction. From indirect evidence Van Harreveld & Stamm (1952) deduced the presence of vascular constriction early in the course of the events that constitute CSD The absolute magnitude of the local cerebral blood flow changes during CSD has neve been reported, nor is the exact temporal relationship between ion concentration changes and cerebral blood flow known.

Since the changes of flow may be related to the extracellular potassium concentration ($[K^+]_e$) (Kuschinsky & Wahl 1978) we investigated the relationship between $[K^+]_e$ and local blood flow

METHODS

Male Wistar rats, in average weighing 360 g, were anesthetized with an initial dose of pentobarbital (50 mg kg⁻¹) and supplementary doses as required later Catheters ere placed in the femoral arteries and one femoral vein. The spontaneously breathing rats were placed in head holder and the parietal bones removed by craniotomy

The experiment were performed as outlined in Fig. 1 Following respiratory steady-state single CSD was elicited from the cortex with brief stab by hypodermic needle through small burr hole in the left frontal bone. The advance of the CSD was monitored with two double barrelled potassium-sensitive microelectrodes with tip diameters of 1– μm. The microelectrodes were passed through the intact dura and placed in the extracellular space of the left parietal corte at depth of approximate

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Presented in part at the meeting of the Scandinavian Physiological Society Odense Denmark, November 1978 (Quistorff et al 1979) and the Symposium on Cerebral Metabolism and Neural Function, U.S National Institute of Neurological and Communicative Disorders and Stroke Bethesda, Maryland, May 1979 (Gjedde et al., 1980b)

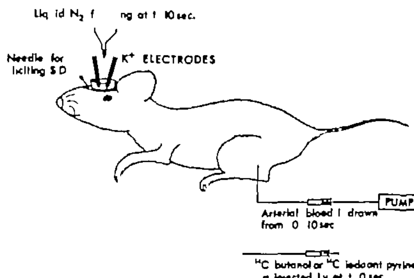


Fig 1 Experimental design A single cortical spreading depres ion (CSD) was elicited by a needle stab in the left frontal cortex The advance of the CSD was followed by two potassium sensitive microeletrodes placed in the left parietal cortex Approximately 30 s after the initial increase of [K]. observed at the posterior electrode regional cerebral blood flow was determined by bolus injection of either [^{14}C]butanol or [^{14}C]iodoantipyrine into a femoral vein and sampling of arterial blood The left and right parietal cortices covered by intact dural membrane were frozen by application of liquid nitrogen after 10 s

ly 0.5 mm (Hansen 1977) A the anterior-posterior dis tance between the electrodes was 3–4 mm the CSD-wa e travelled between the electrodes in one minute (the right parietal cortex was also exposed but no electrodes were inserted)

In order to visualize the blood flow change accompany ing the CSD as well as to quantify the absolute blood flow rate the tissue was freeze-clamped and the di tribution of blood flow was measured by autoradiography and by liquid scintillation counting of small tissue samples

Approximately 30 after the initial change of [K]. detected at the posterior electrode we injected a 200 μl i v bolus of 250 μCi kg 4-[N methyl ^{14}C]-iodoantipyrine for autoradiography (rat) or 10 μCi kg n-[1 C]butanol for tissue sampling (8 rat) and arterial blood sampling was started simultaneously The arterial blood was sampled for 10 s at a known and con tant rate by a yringe attached to a Harward withdrawal pump At the end of this blood collection arrest of the cortical circula tion in itu was produced by generou pplication of liquid nitrogen to the exposed dura o erly ng the parie tal cortex Since only a 0 2 mm wide cleft separates the dural surface from the arteries and em ituated at the surface of the cortex or in the subarachnoid pace freez ing of the blood in the vessel supplying and draining the cortex and thus arrest of the cortical c rculation can be estimated from the result of Qui torff (1980) who showed that the temperature of rat cortical ti sue at a distance of 0 7 mm from a coolant with a temperature of les than −150°C will fall from 37°C to 0°C in 0 8 s It may therefore be estimated that arrest of the cortical circulation was achieved about 1 s after application of the nitrogen and the termination of the collection of blood in the yringe A delay of a few seconds in the arrest of the cortical circula tion would lead to a fall in E(T) cf below for butanol or iodoantipyrine of a few per cent and thus to an underesti mation of blood flow of similar magnitude Howe er in that case the arterial sample collection would have been stopped before the moment of arrest of cortical circulation and the blood flow therefore overestimated due to inser tion of a too low value for $Q_a(T)$ in eq (1) Thus, the two errors introduced by delayed freezing tend to cancel The observed blood flow rates (see later) corresponded very closely to the blood flow rates observed by the same technique in similarly prepared but decapitated rat (Sa & Duffy 1979)

Autoradiography for determination of local cerebr blood flow by labelled iodoantipyrine was performed a follows The frozen brain was removed from the sk under liquid nitrogen and prepared for sectioning in cryostat at −22°C The parietal cortex wa divided into 2 μm tangential sections Every fifth section wa placed o glas slide and rapidly dried on a hot plate (60°) The dried sections were placed face down on the emulsion coated ide of a Kodak Nuclear Medical Blue X ray fi for four days

The autoradiographic density wa measured usi brain cortex section containing a known amount o sotope for calibr tion Identical 0 5 mm brain sect w were cut with a string knife (Franck et al 1967) and sma ch nk (about 20 mg) placed n fl e test tubes containi mock-CSF with add tion of different amount of [C] iodoant pyrine After 30 min the isotope was in equi brium w th the entire lice Twenty μm lices were t cut n a cryostat and some were rapidly dried for densit metry while others were weighed and counted by l

able 1 *Physiological variabl of t included in te present study*

ariable	Mean ± S E.
PaO_2 (mmHg)	95±3
$PaCO_2$ (mmHg)	36±1
Arterial pH	7 41±0 02
MABP* (mmHg)	109±3

Mean arterial blood pressure. Number of observations 10

scintillation spectrometry Films of standard and experimental sections were processed m Beckman densitometer ith beam aperture of 0.2 mm²

T ssue sampling for determination of regional cerebral blood flow by labelled butanol was performed as follows. The frozen brain was divided into 1.2 mm thick coronal slices by an electric saw in glov box at −25°C (Quistorff 1975) The 5 mm wide bits of cortical tissue was isolated eighed and prepared for liquid scintillation counting, as as the arterial sample.

The blood flow of the cortical regions was calculated as described previously (Gjedde et al. 1980) from tissue and arterial blood sample contents of labelled butanol or labelled iodoantipyrine using an equation relating the integrated in- and efflux rates of butanol in brain

$$f^{*} \frac{1}{E(T)} F^{*} \frac{C_M(T)}{Q_A(T)} \qquad (1)$$

m hich E(T) is the net cerebral extraction fraction of radioactive tracer at time T after the introduction of the indicator into the circulation, F* the constant rate of collection of arterial blood $Q_A(T)$ the amount of radioactive tracer collected, and $C_M(T)$ the amount of indicator per unit eight of sample of brain to which f* refers.

The use of eq (1) requires that two criteria are fulfilled: (1) that the time integrals of internal carotid and femoral arterial concentrations of tracer from injection to arrest of cortical circulation are equal, and (2) that efflux of tracer from brain is negligible in the 10 and that therefore E (10) = 1 may be used as good approximation.

With regard to the first criterion, Gjedde et al. (1980a) showed that the integral was independent of the site of sampling from 3 to 30 after injection of the tracer

The second criterion was confirmed by Gjedde et al (1980a) for butanol and by Sakurada et al. (1978) for iodoantipyrine by comparison of cerebral blood flow values calculated with these indicators with values measured directly on the basis of arterial and venous concentrations or by means of inert gases of known, essentially infinite, permeability in the blood-brain barrier

In addition, Crone (1965) showed that the extraction fraction of butanol, integrated from the time of appearance of an intracarotid bolus in cerebral venous blood to time later averaged 0 96 in the dog.

The criteria ere confirmed in rat in which the net extraction fraction of butanol at 10 was measured directly by simultaneous sampling of blood from both

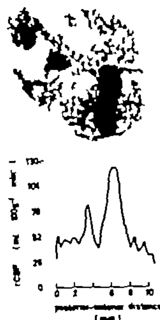


Fig 2 Upper panel Autoradiogram of tangential (horizontal) 20 µm section of rat brain parietal cortex, cut at depth of 0.5 mm A single spreading depression w elicited in the left hemisphere The cortex was frozen in situ by application of liquid nitrogen, ten seconds after an i. bolus injection of labeled iodoantipyrine The frozen cortex was cut in cryostat and the sections placed on an X-ray film for 4 days. The lower part of the autoradiogram represents the left parietal cortex. The spreading depression moved from the right (anterior) part of the section to the left (posterior) one For E(T)~1 the radioactivity of the sample i.e. the grain density is linearly proportional to the local blood flow as shown in eq (1).

Lower panel. Densitometric scan through the left hemisphere of the autoradiogram shown in the upper panel. Densities were converted to blood flow rates by the procedure described in the text.

femoral arteries, one external carotid artery and the superior sagittal sinus of the brain. The amounts of tracer recovered in the superior sagittal sinus and external carotid samples in the 10 s from injection represented 6.5% and 98.3% respectively of the average amount collected in the femoral samples.

RESULTS

The physiological variables of the animals are given in Table 1 The autoradiographs (Fig. 2, upper panel) revealed a remarkable picture An approximately 2 mm wide well-defined band of increased density on the hemisphere where the CSD wave progressed The wide band of increased density

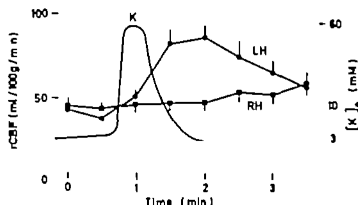


Fig 3 Changes of extracellular potassium concentration and regional cerebral blood flow (rCBF) of 3 mg samples of rat brain parietal cortex during a single cortical spreading depression in the left hemisphere The cortex was frozen in situ by application of liquid nitrogen 10 s after an intravenous bolus injection of radioactive butanol The frozen brain was cut in 1 2 mm thin coronal sections and prepared for liquid scintillation spectrometry The local blood flow was calculated from the radioactivity of each sample The progression of the parietal spreading depression was monitored by potassium-sensitive microelectrodes placed in the cortex of the left hemisphere From the width of the cortical slices and assumption of uniform speed of progression corresponding to that observed between the electrodes spatial flow events were converted to temporal events at the site of one electrode The trace marked K shows the changes of extracellular potassium concentration at one electrode in the left parietal cortex The curve marked LH represents the calculated change of blood flow at the same electrode The curve marked RH represents the change of blood flow at a corresponding site in the right parietal cortex The bars represent one standard error of the mean

was preceeded by a similarly dense but narrow band These areas were separated by a somewhat wider band of normal density The zones of increased density transected the entire cortex

Close examination of the autoradiographs revealed a dotted pattern of increased density in the unaffected cortical regions of the left hemisphere as well as in the cortical regions of the right hemisphere (Fig 2 upper panel) The dots measured approximately 0 4 mm in diameter and probably reflect the presence of cortical columns (Hubel & Wiesel 1962 Mountcastle 1957 Sokoloff et al 1977)

The result of the quantitation procedure is shown in the lower panel of Fig 2 Calculated values for cortical blood flow in the undisturbed parietal cortex varied between 50 and 40 ml (100 g) min the variations corresponding to areas of different densities The scanner aperture of 0 2 mm led to underestimation of the differences in flow in areas with steep density gradients

The local blood flow determined by labelled butanol contents in tissue samples confirmed the picture obtained from the autoradiographs (Fig 3) However the initial narrow band of increased blood flow could not be detected probably becau[se] the lengthwise resolution power of the tissue sam[]pling methods was lower than that of the aut[o]radiogram scanning method (approximately 1.2 m[m] versus 0.5 mm)

The observation that the flow of the undisturb[ed] cortex was 50% of the flow normally observed i[n] rat cortex reflected the barbiturate anesthes[ia] (Gjedde et al 1980*b* Nilsson & Siesjö 1975) Clo[se] scrutiny of the relationship between the [K]e an[d] cortical blood flow revealed the presence of thre[e] phases Initially the [K]e increased to 60 m[M] while the cortical blood flow remained unchanged During the second phase cortical blood flow [in]creased to twice the normal value while [K]e re[]turned to 3 mM During the final phase whi[ch] lasted about one minute cortical blood flow de[]creased to its previous level and no further chang[e] of [K]e was noticed

DISCUSSION

The present study demonstrates for the first tim[e] the marked change in cerebral blood flow occurrin[g] during a single CSD in the rat The CSD represent[s]

phenomenon in which significant variations of $[K^+]_e$ and blood flow occur in the cerebral cortex. Therefore the phenomenon is ideally suited for a study of the relationship between the two variables. A prerequisite for this study was the development of a method which can detect rapid flow changes (Gjedde et al. 1980).

It is evident from the present study that high cerebral blood flow was not directly associated with the increased $[K^+]_e$ but rather with the subsequent reduction of $[K^+]_e$. It is well known that increased cerebral blood flow is coupled to increased metabolic activity in the brain (Reivich et al. 1975) and that high $[K^+]_e$ stimulates the metabolic rate of nervous tissue (Ashford & Dixon 1935, Hertz & Clausen 1963, Lothman et al. 1975, Shinohara et al. 1979). Thus, it is likely that the effect of increased $[K^+]_e$ on cerebral blood flow is mediated through metabolic factors that may be related to increased ion pumping in connection with the return of potassium to the intracellular compartment (Quistorff et al. 1979). The metabolic factor(s) that constitute(s) the link between metabolism and flow remain(s) to be identified.

In the past, several factors including O_2, H^+, K^+ and adenosine have been implicated in metabolic autoregulation. However, it has not been possible to demonstrate parallel changes of any of these factors in all the conditions of increased blood flow investigated (Astrup et al. 1976, Leniger-Follert & Lübbers 1979, Rubio et al. 1975).

The flow variations during a CSD can partially be explained by an action of potassium itself on cerebral vessels. It has been shown that an increase of $[K^+]_e$ up to 10 mM caused dilatation of pial vessels while further increases lead to constriction (Kuschinsky & Wahl 1978). Thus, a marked transient increase of $[K^+]_e$ would initially induce dilatation of cerebral vessels, followed by a vasoconstriction. Subsequently cellular re-uptake of K^+ (and extrusion of Na^+ (Hansen 1979)) could give rise to metabolically-induced vasodilatation. This succession of events was actually observed in the autoradiographs obtained in this study. Thus, the first peak of increased density corresponds to the initial increase of $[K^+]_e$, followed by vasoconstriction (the intermediate band of decreased density), ending in a marked longer lasting vasodilatation, during which K^+ is pumped back into the cells.

The present data suggest that the major perturbation of cerebral blood flow occurs after the wave of $[K^+]_e$ increase. The cellular events immediately preceding the changes of extracellular ion concentrations during CSD are still unknown.

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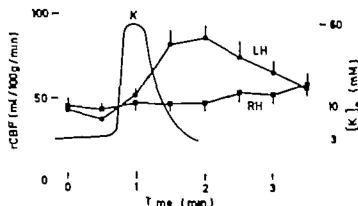


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The observation that the flow of the undisturbed cortex was 50% of the flow normally observed in rat cortex reflected the barbiturate anesthesia (Gjedde et al 1980*b* Nilsson & Siesjö 1975) Close scrutiny of the relationship between the [K]e and cortical blood flow revealed the presence of three phases Initially the [K]e increased to 60 mM while the cortical blood flow remained unchanged During the second phase cortical blood flow increased to twice the normal value while [K]e returned to 3 mM During the final phase which lasted about one minute cortical blood flow decreased to its previous level and no further change of [K]e was noticed

DISCUSSION

The present study demonstrates for the first time the marked change in cerebral blood flow occurring *during a single CSD in the rat The CSD* represents

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Oxygen supply oxygen consumption and endocrine and exocrine secretions of the isolated, perfused porcine pancreas

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HOLST J J LINDKÆR JENSEN S NIELSEN O V & SCHWARTZ, T W Oxygen supply oxygen consumption and endocrine and exocrine secretions of the isolated, perfused, porcine pancreas Acta Physiol Scand 1980 109 7 13 Received 14 July 1979 ISSN 0001-6772. Institute of Medical Physiology C University of Copenhagen, Department of Surgery C, Rigshospitalet Copenhagen and the Institute of Medical Biochemistry Aarhus University Denmark.

W studied the effect of varying oxygen supply on the endocrine and xocrine secretions of the isolated perfused porcine pancreas utilizing completely synthetic perfusion medium with and without the addition of erythrocytes. With ynthetic medium o ygenated to a P of 500 mmHg, oxygen consumption was constant for flow rates at or above 0.5 ml min^{-1} g^{-1} (et weight). Addition of erythrocytes to the medium did not increase oxygen consumption at flow rates above this level Furthermore the secretion of fluid bicarbonate and protein in response to secretin and acetylcholine was not influenced by addition of erythrocytes Similarly the secretion of insulin glucagon, pancreatic polypeptide, and somatostatin in response to arginine and acetylcholine was unchanged arginine stimulated the secretion of all four peptides, whereas acetylcholine stimulated the secretion of insulin and pancreatic polypeptide and inhibited glucagon and somatostatin secretion. The results indicate that the porcine pancreas is respiring adequately when perfused with completely synthetic perfusion medium at flow above about 0 5 ml × min g and P_{O_2} about 500 mmHg, and that addition of erythrocytes is not necessary for the study of its secretory functions.

The isolated perfused pancreas has been used extensively in studies of pancreatic endocrine and exocrine secretion (Ross 1972 Scratcherd & Case 1973) In our studies of the *secretory effects of* gastrointestinal hormones and transmitter substances on the pancreas (Lindkær Jensen et al. 1978a, b 1979 Schwartz et al. 1978) we found it essential to make use of the isolated perfused pancreas, in order to avoid neural or endocrine effects other than those under study The porcine pancreas was chosen because the majority of the gastrointestinal hormones are known in their porcine form only *The* oxygen supply to the isolated perfused organ preparation is usually provided either by oxygenation of synthetic perfusion medium to a high partial pressure of oxygen, or by the addition of erythrocytes to the medium before oxygenation However a comparison between the two methods, in which endocrine as well as exocrine secretions are considered, has not been carried out.

In the present report such a study is described

METHODS AND MATERIALS

The pancreatic glands from 13 young pigs of Danish landrace (appr 25 kg) were studied Isolation and perfusion was performed as previously described (Lindkær Jensen et al 1976 Lindkær Jensen et al 1978) In short, the glands were isolated together with 12 cm segment of the aorta, comprising the celiac trunck as well as the superior mesenteric artery The duodenum was not included in the

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ble Oxygen consumption (μl O × g × min) at variou flow rat s ith or ithout addition of erythrocyte t the perfusion med m

each of 6 perfusions, mean values based on 1-4 duplicate determinations were calculated and these data are here mmarized as mean ± S E. of the 6 perfusions

	Flow rate (ml g)		
	0.30±0 01	0 48±0 02	0 72±0 03
ithout erythrocytes	3 18±0 19	6 31±0 51	7.05±0.36
ith erythrocytes	7.39±0.43	8.84±0.52	7 47_0.5

ach basal level of secretion again The medium was en supplemented with erythrocytes and the entire pro-ol was repeated except that the flow changes were ade after the timulation periods. In 3 expts. the gland ere perfused ith erythrocytecontaining medium first nd ynthetic medium later

11 7 glands ere perfused with synthetic medium only flow rates anging from 0 37 to 0 63 ml min g for 40 min and ygen consumption determined every 30 m

nal ses

concentrations in pancreatic juice of protein and bicarbo-ate and in effluent of insulin glucagon and pancreatic olypeptide were measured as previously described Lindkær Jensen et al 1978a Schwartz et al. 1978). omatostatin concentrations in effluent were measured by adioimmunoassay The antiserum, R 213 was raised in a abbit against synthetic somatostatin covalently bound to ovine serum albumin (Rehfeld et al., unpublished tudies) Standards were ynthetic, cyclic somatostatin Beckman Bioproducts, Geneva, Switzerland), and the acer as ¹²⁵I-tyr-somatostatin Labelled according to tuanura (1975) (a generous gift from NOVO Research Institute Bagsværd, Denmark) Detection limit of the assay as 1 pmol/l, interassay coefficient of variation better than 6% in the working ange Bound and free somatostatin were separated with plasmacoated charcoal The antiserum react equally ell with reduced and cyclic omatostatin and also bind equally well ¹²⁵I-somatostatin with tyrosin substituted in position 1 and position 11 The antiserum does not crossreact with other known pancreat peptides The results of recovery studies performed on he effluent from the perfused pancreas deviated less than % from the expected values.

alculations

The hormone outputs were calculated on the basis of concentration determinations in all effluent samples. The total output during stimulation was calculated as the sum of the 1-min outputs The hormone outputs in the immediately preceding 10 or 5 min periods were calculated in the same way for comparison (Fig 3). I Fig. 3 the data are presented as (average) outputs per min. Statistical evaluation as performed by means of Student' -test for paired data The level of statistical significance was chosen as 0 05

RESULTS

Influent and effluent values of P_{O_2}, pH and P for the 6 gland which were perfused with and without erythrocytes appear in Table 1 and oxygen consumption values calculated on the basis of a coefficient of solubility for oxygen in perfusion medium of 0 023 and oxygen binding capacity of hemoglobin of 1.39 ml×g are shown in Table 2. Influent P values were slightly lower with erythrocytes than without and ranged between 400 and 500 mmHg Without erythrocytes oxygen consumption was significantly lower at a flow rate of 0.3 ml × min × g than at 0 48 ml × min × g but was not increased by raising the flow rate to 0 72 ml × min × g In agreement with this observation, the effluent P_{O_2} increased from 60±3 to 204±18 mmHg by the latter increase in flow rate To substantiate further that the maximum oxygen consumption was reached at a perfusion rate of approximately 0.5 ml × m n × g we determined

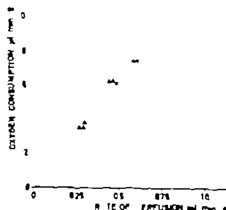


Fig 1 The relationship between oxygen consumption and rate of perfusion without erythrocyte addition. The filled circles (●) represent the mean of 6 determinations in each of seven perfusion experiments The data indicated by ▲ are the individual mean values from Table

Table 1 P_{O_2}, pH and P_{CO_2} values from the isolated perfused porcine pancreas

Influent (A) and effluent (V) P_{O_2}, pH and P_{CO_2} from perfusion of 6 isolated pancreatic glands For each pancreas mean values based on 1–4 duplicate determinations were calculated and these data are summarized as mean followed by SE of the 6 perfusions

Flow (ml × min⁻¹ × g⁻¹)	Sample	P_{O_2} without erythroc	P_{O_2} with erythroc	pH without erythroc	pH with erythroc	P_{CO_2} without erythroc	P_{CO_2} with erythroc
0 30±0 01	A	440±12	397±59	7 45±0 01	7.36±0 02	36 4± 3	43 ±3
	V	48± 7	3 ± 9	7 18±0 0?	7 21±0 03	61 4± 6	58 ±3 8
0 48±0 02	A	488±15	424±40	7 41±0 02	7.39±0 0	34 7±4 0	38.8±3 8
	V	60± 3	41± 5	7 25±0 02	7 25±0 04	50 3±? 4	53 4±5 4
0 72±0 03	A	514±12	438±68	7 44±0 01	7 4 ±0 07	41 8±4 6	39 9±6
	V	204±18	182±45	7 25±0 0	7.21±0 01	54 8±3 8	54.3±4

preparation but the pancreatic-duodenal vascular supply to the pancreas remained intact Thus the pancreas was perfused through its entire arterial supply i e the splenic the gastroduodenal and the superior mesenteric arteries, and drained through its entire contributions to the portal vein

The pancreatic gland were perfused at 38°C with Krebs-Ringer bicarbonate solution supplemented with fumarate glutamate and pyruvate each 5 mmol/l Dextran T 70 (Pharmacia Fine Chemicals Uppsala Sweden) 40 g/l human serum albumin (trocken reinst Behringwerke GFR) 1 g/l and glucose 3.5 mmol/l The perfusate was oxygenated with 96% oxygen 4% CO_2 using a multiple bulb oxygenator (Ross 1972b) and the flow was controlled by an adjustable finger pump (Ole Dich Copenhagen) Perfusion pressure did not exceed 50 mmHg The effluent was collected for 1 min periods by means of an automatic fraction collector centrifuged (when erythrocytes were present) and frozen immediately at −20°C The pancreatic duct was cannulated with a polyethylene catheter (dead space 150 µl) and pancreatic secretions collected for 5 or 15 min periods and frozen

Influent and effluent samples for gas analysis were obtained close to the pancreas every 10–30 min in gas tight syringes under anaerobic conditions and analysed for P_{O_2} P_{CO_2}, pH HCO_3 hemoglobin concentration and saturation of oxygen using the Acid Base Laboratory II Radiometer Copenhagen Denmark and standard oxygen electrodes (Radiometer Copenhagen) Oxygen consumption was calculated as flow × the arteriovenous concentration deficit of physically dissolved and chemically bound oxygen

Pancreatic exocrine secretion was stimulated by addition of secretin (pure porcine secretin GIH Research Laboratories Stockholm Sweden) to the influent for 5 min (by means of a side-arm pump) to a final concentration of 300 pmol/l or by acetylcholine (also 5 min) (Sigma Chemical Company Miss USA) 10^{-6} mol/l Endocrine secretions were stimulated by addition of arginine 5 mmol/l for 10 min

Erythrocytes were prepared from outdated human bank blood stored for 3 weeks in Citrate Phosphate Dextrose the erythrocytes were washed several times with saline and perfusion buffer filtered and added to the perfusion medium reservoir to a hematocrit of 10% pH was—if necessary—adjusted by means of $NaHCO_3$ 0 1 mol/l

Experimental protocol

I 6 glands After 30 min perfusion (equilibrium period) at about 0.5 ml/g × min with medium without erythrocytes the flow rate was adjusted to 0 48±0 02 ml/g × min 0 30 ±0 01 ml/g × min 0 72±0 03 ml/g × min and again 0 48 ml/g × min each period lasting 10 min

The glands were then stimulated with the following agents at a flow of (approximately) 0 5 ml/g × min arginine 10 min followed by (at least) 15 min rest acetylcholine 5 min and 10 min rest secretin for 5 min and min rest We knew from previous experience that the rest periods were of sufficient length for the glands

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The ABL-II (Radiometer Copenhagen Denmark) is an automatic bloodgas analyser which measures pH P_{CO_2} P_{O_2} and hemoglobin concentrations and calculates bicarbonate standard bicarbonate total CO_2 and—by solving a Hill-equation with a Bohr factor of −0 48 and a P_{50} of 26 6 mmHg—calculates oxygen saturation However the machine cannot reliably measure the P_{O_2} of solutions which do not contain hemoglobin Therefore determinations of P_{O_2} in medium without erythrocytes were performed with a standard oxygen electrode as well Furthermore by comparing the hemoglobin determinations of the ABL-II with results from conventional method on identical samples of erythrocyte-containing perfusate it was found that the former exceeded the latter by a factor 1.31 hemoglobin values were corrected accordingly Finally the P_{50} for the hemoglobin binding of oxygen used in the calculations 26 6 mmHg is probably higher than the P_{50} of erythrocytes stored for 3 weeks in CPD which has been reported to be approximately 20 mmHg (Hursmar et al 1969) therefore all oxygen saturations were calculated using the following equation

$$S = 100 \times \frac{Z^{3}}{(20\ 0)^{3} + Z^{3}} \quad \text{where}$$

$$Z = P_{O_2} \times 10^{-0.48(\ \ \ )}$$

This equation is identical to the one used by the ABL-II apart from the substitution of 26 6 with 20

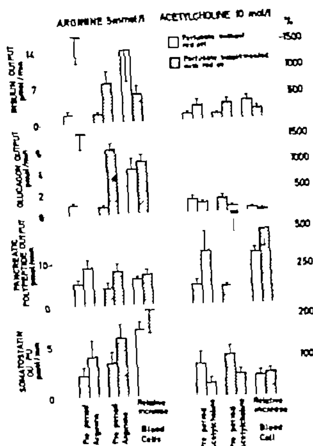


Fig. 3. Endocrine secretion of the isolated porcine pancreas, perfused with or without erythrocytes. The columns represent mean ±S.E. (=6) or hormone outputs before and during stimulation. I addition, the relative response to stimulation—given as output during stimulation in percentage of basal output—is shown in the right hand columns.

gen consumption of an isolated pancreas per ed with a synthetic medium. The only way to mine whether the oxygen requirements are met vitro is to study the relationship between flow e and oxygen consumption the flow rate above ich the oxygen consumption cannot be increased ther should then provide sufficient oxygen for preparation. A flow of this limiting magnitude ves no room for full coverage of oxygen re- irements during stimulation and to increase rther the oxygen supply by larger increases in w rate may apart from being prodigal of perfu- on medium, be inexpedient, since at high flow tes with synthetic medium there is a risk of edema rmation. Furthermore, with the pancreas, the fact at the majority of the tissue the exocrine gland, respires adequately does not provide a guarantee that the endocrine gland is also adequately per fused.

To solve these problems we studied the exocrine and endocrine secretions of the isolated porcine pancreas in response to various stimuli during per fusion with and without erythrocytes by addition of erythrocytes the oxygen supply could be increased without increasing the flow rate. Our results show that at or above a flow rate of 0.5 ml × min × g and with an influent oxygen tension of about 500 mmHg, the oxygen consumption of the pancreas could not be increased further (in the basal state) by increasing flow rate and the chosen flow rate should therefore ensure adequate oxygenation under such conditions. Furthermore addition of

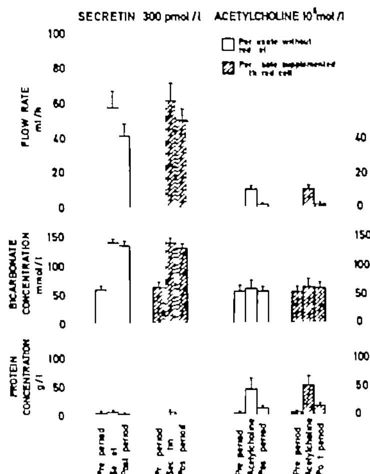


Fig 2 Exocrine secretion of the isolated pancreas perfused with or without erythrocytes. The columns represent mean ± S E. (*n*=6) of flow of juice bicarbonate and protein concentrations before during and after stimulation

the oxygen consumption of further seven glands perfused at flow rates ranging from 0 37 to 0 62 ml × min × g The combined results are shown in Fig 1 There is a nearly linear relationship between oxygen consumption and flow rate up to approximately 0 5 ml × min × g above this flow rate oxygen consumption appears to remain constant During perfusion with erythrocytes oxygen consumption remained constant over the flow range tested (Table 2) and identical to that obtained during perfusion at 0 72 ml×min ×g at 0 48 ml × min × min oxygen consumption was 40% higher with than without erythrocytes

The basal exocrine secretion was uninfluenced by erythrocyte addition The exocrine responses to secretin and acetylcholine appear in Fig 2 secretin stimulated flow and bicarbonate secretion and the results are comparable to previously reported results (Lindkær Jensen et al 1978*a*) Acetylcholine stimulated flow and protein secretion significantly Virtually identical results were obtained with and without the addition of erythrocytes

The endocrine secretions appear in Fig. 3 Arginine significantly stimulated the secretion of all pancreatic hormones whether erythrocytes were present or not and the stimulated outputs relative to the basal outputs were not significantly different Acetylcholine significantly stimulated insulin and pancreatic polypeptide secretion and significantly inhibited glucagon and somatostatin secretion Similar results were found after addition of erythrocytes The concentration variations in a single typical experiment are shown in Fig 4

DISCUSSION

Because of the complexity of the venous drainage of the pancreas it is difficult to measure its oxygen consumption in vivo the investigator is therefore left with some uncertainty when evaluating the

…etylcholine on somatostatin secretion has not … reported before: lower concentrations of …ylcholine however have no effect and the …onse to 10^{-} mol × l^{-} is probably not a …iological action since this dosage (*a*) is far …ve the D_{50} for e.g. stimulation of pancreatic …peptide secretion (Schwartz et al. 1978) and (*b*) …s to an increase of the vascular resistance of …creas (unpublished studies). The effects of …tylcholine on the exocrine secretion of the iso…d porcine pancreas have not been studied be… and the results are of great interest in view of … marked effects of vagal stimulation on the pan…atic exocrine secretion in the pig (Hickson 1970 …st et al. 1978). In contrast to the effect of …al stimulation, however, acetylcholine does … affect the concentration of bicarbonate in the …e secreted by the isolated perfused pancreas. …se findings are in agreement with the …otheses, that the vagally induced bicarbonate …retion is not entirely mediated by cholinergic …smission (Holst et al. 1978, Fahrenkrug et al. …9).

…he present results support the conclusion, put …ward in our previous reports (Lindkær Jensen …8 … *b*) that the isolated porcine pancreas, per…ed with a completely synthetic medium at P… of …) mmHg is a reliable tool in the study of pan…atic endocrine and exocrine physiology.

…gratefully acknowledge the invaluable technical assist…e of Bente Hansen Nielsen, Lathy Klarskov, Jindra …rbyerg, Lone Jørgensen and Hanne Nordvig. W… …nk the department of internal medicine A, Rigshos…alet for making the ABL-II accessible to us. This study …s supported by grants from "Den lægevidenskabelige …tilskudsfond for Storkøbenhavn, Færøerne og …ønland" and Landsforeningen for sukkersyge.

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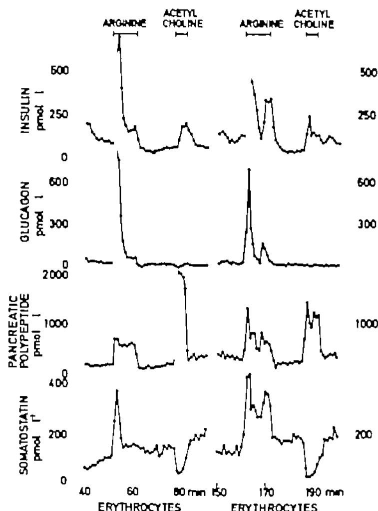


Fig 4 Hormone concentrations in the effluent from an isolated perfused pancreas in response to arginine and acetylcholine with and without erythrocytes in the perfusate Results of a single typical experiment

erythrocytes during the higher flow rate—still in the basal state—did not increase oxygen consumption further

The oxygen consumption during perfusion at a mean flow of 0 48 ml × min × g with erythrocytes did not differ from those obtained at the higher and lower flow rates but differed slightly and significantly from that obtained without erythrocytes indicating that the flow rates below the average value of approximately 0 5 ml × min × g were insufficient to ensure complete oxygenation Nevertheless the endocrine and exocrine secretions basal as well as stimulated were comparable whether erythrocytes were added or not Therefore perfusion with a completely synthetic medium without erythrocytes at a flow which must be maintained at at least 0.5 ml × min × g (or somewhat above) and P about 500 mmHg appears adequate for the study of the endocrine and exocrine secretions of the porcine pancreas

Some of the secretory results deserve comment The effect of acetylcholine on insulin secretion agreement with previous reports (Iversen 19 Loubatières-Mariani et al 1973) on cholinergic effects on rat and dog pancreas In the present experiments however acetylcholine did not stimulate glucagon secretion (on the contrary a significant inhibition was noted) in agreement with our previous observations in pigs (Holst et al 1977) In dog acetylcholine stimulates glucagon secretion (Iversen 1973 Kaneto & Kosaka 1975) also in our hands acetylcholine at 10 mol/l stimulates the secretion of glucagon from the isolated perfused dog pancreas (Holst & Lindkær Jensen unpublished studies Undoubtedly we are facing important species differences (Larsson et al 1978) An inhibitory eff

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epression of mechanical performance by active shortening uring twitch and tetanus of vertebrate muscle fibres

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EDMAN K. A P Depression of mechanical performance by active shortening during twitch and tetanus of vertebrate muscle fibres. Acta Physiol Scand 1980, 109 15–26 Received 19 July 1979 ISSN 0001-6772. Department of Pharmacology University of Lund Sweden

Shortening during activity of frog single muscle fibres caused graded depression of the contractile force that persisted for 800–900 ms during a partially fused or completely fused tetanus. The depression of force was not associated with change of the shortening velocity at zero load. Passive shortening performed just before stimulation had no effect on the subsequent course of contraction. The decrease in isometric force produced by shortening wa not significantly affected by a twitch applied immediately before or after the shortening phase. For a given amount of shortening the depressant effect during a fused tetanus was 8–28% of that produced during twitch. The effect was substantially reduced, both during twitch and tetanus in the presence of 0.5 mM caffeine The length dependence of the movement effect was studied between 1.7 and 2.9 μm sarcomere spacing. Maximum depression of force (in per cent of the control at each length) was obtained at 2.1–2 μm sarcomere length the effect being steadily reduced at shorter and more extended lengths The Q_{10} of the depressant effect was 0.95±0.16 (S.D). The features of the movement effect are consistent with a true deactivation of the contractile system as would occur if shortening reduced the binding of activator calcium to the regulatory proteins of the myofilaments.

Key ords Skeletal muscle fibres, force depression by shortening, shortening induced deactivation

ertebrate skeletal muscle which is allowed to sorten during a twitch loses some of its contractile rength during the remainder of the activity period ~well & Wilkie 1960 Hill 1964 Joyce & Rack 69 Joyce Rack & Westbury 1969 Edman & essling 1971 Briden & Alpert 197? Dickinson & okedge 1973) A more detailed study of this phe imenon during twitch and partially fused tetanus is previously been performed on isolated frog uscle fibres (Edman 1975) It was shown that e depressant effect is long-lasting and does not sappear merely by restimulation of the fibre It as furthermore demonstrated that the effect is t significantly dependent on the load, or the ork output during the shortening phase These ndings argue against the idea that the reduced nsion development after the movement is due enhanced re-uptake of activator calcium by the rcoplasmic reticulum or to exhaustion of some ergy substrate for the contractile process (cf ill 1964) It was suggested, as a more plausible explanation of the movement effect (Edman 1975) that shortening causes a change in the myofilament system itself that leads to a reduced degree of interaction between the A and I filaments

The present investigation extends the previous study of the movement effect to include recordings also during a fused tetanus. Experiments have been designed to investigate if the depressant effect is dependent on the area of overlap between the A and I filaments the temperature and the degree of activation of the contractile system when the movement occurs Attempts have also been made to find out if the decrease in isometric force is associated with a similar change of the shortening velocity at zero load. Some of these results have been reported in a preliminary form (Edman 1976)

METHODS

Preparations. Single fibres from the semitendinosus and tibialis anterior muscles of Rana temporaria were studied. The essentials of the techniques used for dissection and

...le 1 *Comparison between depressant effect of active shortening during single twitch, partially fu ed ... completely fused tetanus*

	Amplitude of shortening in excess of control (μm/sarcomere)	Depression of peak force (% control)		
		Single twitch	Partially fused tetanus	Fused tetanus
1975	0.46		40.5	7.5
...t 1976	0.16	29.7		4.0
...n 1976	0.20	47.5		5.2
1977	0.14		24.7	7.0
1979	0.19	48.4		4.4
...1979	0.16	51.3		4.0

...ngth was adjusted to 2.25 μm. The measurements of the ...ross-sectional area was performed in separate trough ... 500 magnification as previously described (Edman ...75).

...Recording and measurement of responses. Tension and ...ength changes were displayed on Tektronix 5103N ...orage oscilloscope and photographed on 35 mm film. ...easurements from the film records were made in ...kon model 6C profile projector at 10 magnification ...dman 1979).

Velocity of unloaded shortening. The velocity of short...ning at zero load, V_0 was measured using the multiple ...lease method described previously (Edman 1979). As ...monstrated in the previous paper V_0 is constant be...ween 1.65 and approximately 2.7 μm sarcomere length. ...e measurements in the present study were performed ...tween 1.80 and 2.0 μm sarcomere spacing as exempli...ed in Fig. 7.

...ESULTS

Comparison of force depression by shortening ...ring twitch and t tanus

...he approach used for determination of the depres...ant effect of active shortening was similar to that ...reviously described (Edman 1975). The contract...on was initiated at approximately 2.4 μm sarco...ere length and the fiber was released during con...raction to shorten and redevelop force at a new ...ength between 2.25 and 1.95 μm, i.e. within a range ...here the tetanic tension is virtually constant. Two ...mplitudes of shortening were used. The smallest ...elease (the control) was sufficiently large to pro...uce a complete drop in tension immediately fol...owed by redevelopment of force. With the larger ...elease (the test) the fibre was allowed to shorten ...ver an additional distance before it started to ...edevelop tension. The releases were timed so that ...e redevelopment of tension occurred at the same moment during the activity period in both test and control. The speed of release was high enough to prevent force production during the shortening phase.

Fig. 1 illustrates release recordings performed during a single twitch (A) and a fused tetanus (B) in the same fibre. It can be seen that the extra amount of shortening during the test release (0.16 μm/sarcomere) reduced the rate of rise and the peak amplitude of the redeveloped force. The time to peak tension was not markedly affected (see also Fig. 2, Edman 1975) but there was a certain (approximately 15%) reduction of the time to half relaxation. The depression of peak force, in per cent of the control, was 29.7% during the single twitch. A considerably smaller (4.0%) depression was obtained when the same amount of shortening was produced during the plateau (prior to the last stimulus) of the fused tetanus. Table 1 summarizes the results of six experiments similar to that illustrated in Fig. 1. In these experiments a sarcomere shortening of 0.14–0.46 μm was required to reduce the peak redeveloped force by 4.0–7.5% during a fused tetanus. The same amount of movement performed during the rising phase of a single twitch or during the last cycle of a partially fused tetanus reduced the peak redeveloped force by 25–51% in the same fibres.

The depressant effect of shortening during a fused tetanus is further illustrated in Fig. 2. In this experiment the stimulation was continued for about 800 ms after the end of the shortening. It can be seen (inset, Fig. 2) that the speed of force redevelopment (dF/dt) was lower at any given tension level during the first few hundreds of milliseconds after the large movement. The value of dF/dt at 50%

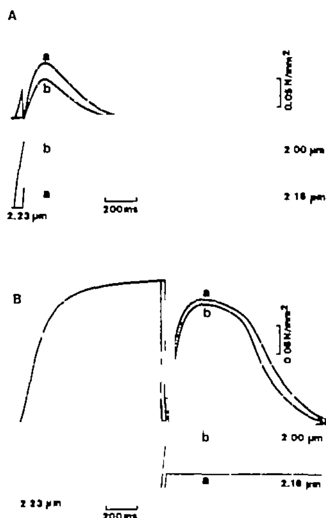


Fig 1 Depressant effect of acti e shortening during a single twitch (A) and a fused tetanus (B) in an isolated muscle fibre The time of release was adjusted appropriately to allow the fibre to redevelop ten ion at nearly the same time after the small (0 07 μm sarcomere myogram *a*) and the large (0 ‿3 μm/sarcomere myogram *b*) movement. Lower traces position of puller arm indicating sarcomere length before and after the shortening. Note (1) that the force redevelopment is smaller after the large movement and (2) that the reduction of force is more pronounced during the twitch than during the fused *tetanus.* Temperature 0°C Cross sectional area 11 589×10 mm

mounting of the fibres have been described previously (Edman 1975 1979) The fibres were mounted horizontally in a jacketed Perspex bath between a force transducer and the tip of a shaft which was connected to the mov ing coil of an electromagnetic puller The rest length of the preparation could be set to the desired value by adjusting the position of the puller or the tension trans ducer

Sol tion The bathing solution had the following com position (mM) NaCl 115 5 KCl 2 0 $CaCl_2$ 1.8 Na phosphate buffer ‿0 pH 7 0 The solution (8 ml) was ex changed before each series of contraction The tem ture was controlled by circulating a water-glycol sol from a thermostatically controlled tank (Colora Ultr mostat) through the jacket surrounding the chamber bath temperature was maintained constant to during the expt the temperature varied between .5°C between different expts

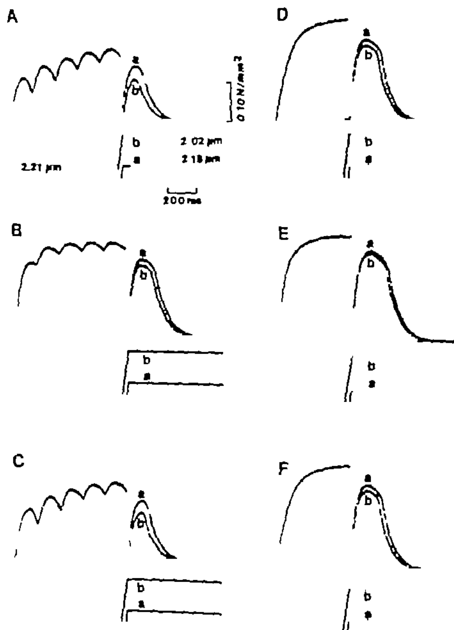
Stimulati n The fibres were stimulated by current through two platinum plate electrodes place either side of the fibre approximately mm fr Rectangular pulses of 0‿ ms duration were used stimulus strength was approximately ‿5 above th old A single pulse a series of 4-6 pulses of 3-5 Hz quency or a 1 s train of pulses of a frequency of Hz were given to produce a single twitch an incomp ly fused tetanus or a fused tetanus respectively Con tions were produced at min interval during the expt The fibres were usually mounted f r -4 h be the expt began and were tetanized periodically during time The contractile performance was usually stable a whole day of experimentation Deterioration of the f was detected by decreased rate of rise of tension the fused tetanus tension drop during the tetanus pl and/or increased dispersion of sarcomere length flected by a widening of the first-order line of the diffraction pattern

Tension recording An RCA 5734 mechano-ele transducer was used with a tubular glass extension hook for connecting the fibre The compliance of the extension and hook was 1 5 μm/mN The natural quency of the tension transducer wa approximate kHz when the glass shaft was immersed in solution later experiments a semiconductor strain-gauge tr ducer (AE 801 Aksjesel Kapet Mikroelektronikk) used A hook of tainless steel wire was attached by re in to the tip of the transducer bar The resonant quency of the transducer submerged in the solution about 5 kHz There was no detectable creep of the after a sudden unloading of the transducer

Electromagnetic pulle ervo ystem and displace tran du r These were essentially the same as descr pre iously (Edman 1975) The release movements performed from the same starting point of the el magnetic coil In order to attain a given final leng the fibre after different steps the re t length of the was adjusted appropriately This could be done with accuracy of 5 μm (0 1 % of the fibre length Edman 19 For illustration of such an expt (Fig) the record the displacement transducer ha e been shifted ph graphically to show the actual sarcomere length be and after the release step

S rcomere length ord g The sarcomere lengt rest and during activity w determined from the la d ffraction pattern The change in sarcomere length occurred in esponse to a release during activity calculated from the change in overall fibre length and sarcomere length t the onset of the release movement

Determ nat n of fibre l gth d sect nal ar The length between the insertions of the fibre to the dons was measured to the nearest 0 05 mm with a Z Stereo II microscope (6 or 10 magnification) at the of each expt. Before this measurement the sarcom



r 3 Influence of caffeine on the depressant effect of active shortening. Control ( ) and test (*b*) movements performed ring the last cycle of partially fused tetanus (A–C) and prior to the last stimulus of fused tetanus (D–F). A, D: fibre mersed in ordinary Ringer solution. B E, 45–65 min after addition of 0.5 mM caffeine to bathing fluid C, F 50–65 n after change to ordinary Ringer solution Lower traces indicate sarcomere length before and after the shortening. e first standies marker in A and the first two markers in D have been retouched Temperature 2.5°C. Cross-sectional ea 4 817 10^{-9} mm²

yogram c the contraction was initiated at 2.20 µm d the fibre was allowed to shorten to 1.85 µm ter which it was stretched back to its original ngth The fibre's ability to produce force after a complete drop in tension was tested at various times during cycles 2–6 in runs where no shortening (or stretch) had been performed during the first two cycles. For this purpose a small release (0 09

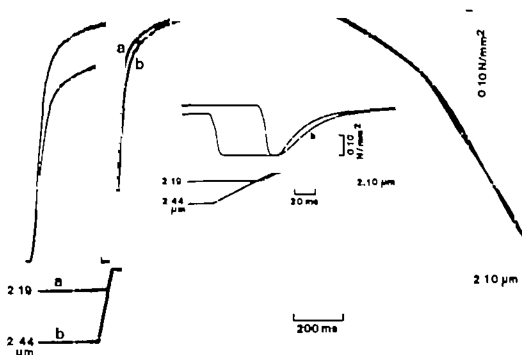


Fig 2 Superimposed oscilloscope records illustrating redevelopment of tension after small (*a*) and large (*b*) shorte during a fused tetanus Inset recordings on fast time base Lower records, position of puller arm indicating sarcom length before and after the shortening Temperature 1 2°C Cross sectional area, 13.508×10⁻ mm

of maximum tetanic force was 17% lower after the large movement than after the small control movement The fibre regained its ability to produce force however so that almost the same tension level was attained after the large and the small movement at the end of the tetanus

2 *The influence of caffeine on the movement effect*

The finding that shortening caused a smaller depression during a fused tetanus than during a single twitch or a partially fused tetanus suggested that the movement effect might be related to the degree of activation of the contractile system when the shortening occurs As an attempt to further elucidate this possibility the depressant effect was studied after addition of 0 5 mM caffeine to the Ringer solution This agent is likely to increase the amount of activator calcium in the myofibrillar space in response to excitation of the fibre membrane (e g. Ebashi 1976 Blinks Rudel & Taylor 1978)

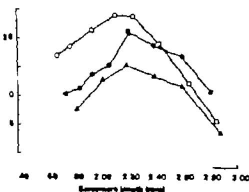
Fig 3 A–E shows that shortening caused a considerably smaller depression of the isometric force after 0 5 mM caffeine had been added to the bath The depressant effect was 25% during the last cycle of the partially fused tetanus and 7% during the completely fused tetanus in the ordinary Ring solution These effects were reduced to 7 and 3 respectively when the shortening was carried in the presence of caffeine Removal of the caffe restored the original magnitude of the movem effect (Fig 3 C F) Results similar to those illu trated in Fig 3 A–F were obtained in three oth expts

3 *Lack of effect of passive shortening*

Shortening of the fibre *before* activation had detectable effect on the subsequent course of co traction This was tested by allowing the fibre shorten passively from 3 05 to 2 60 µm sarcomer length immediately before the stimulus was applied As can be seen in Fig 4 twitches recorded at µm with and without preceding passive shorten were identical in shape

4 *Tension development after shortening-restretch*

Attempts were made to establish if restretch of the fibre after shortening reverses the depressant effe produced during the shortening phase Fig 5 illustrates such an expt Here are shown three my grams of partially fused tetani at 20 µm sarcomer length In myogram *b* the fibre was allowed shorten from 55 µm during the first cycle I



6 Depressant effect of active shortening recorded various sarcomere lengths in three different fibres. scissa sarcomere length at which redevelopment of tion was measured after shortening Ordinate: Depressant effect expressed in per cent of the control peak force each length. Sarcomere shortening during test run, in cess of control release. O 0 24 μm/sarcomere ● 28 μm/sarcomere, Δ, 0.23 μm/sarcomere. Temperature id cross-sectional area, O 1 1°C 8 967 10⁻⁴ mm² 0.6°C 8 980 10⁻ mm² Δ, 1 9°C 5.604 10⁻ mm²

m/sarcomere) was carried out at selected times nd the peak redeveloped force was recorded at 20 μm sarcomere length. These data points (filled rcles Fig 5 A) serve as controls for estimation f the amount of force depression during cycles 3–6 n myograms *b* and *c*

The results in Fig. 5 A show in confirmation of previous results (Edman 1975) that shortening during the first cycle caused a marked depression of the contractile strength and that this effect gradually disappeared over a time period of approximately 800 ms, i.e by the attainment of peak tension during the sixth cycle Restretch of the fibre after an initial shortening phase did not reverse the depressant effect. In fact myogram *c* exhibits a somewhat larger force depression than myogram *b* This difference may be due to the fact that the shortening in myogram *c* occurred over a range of sarcomere lengths where the depressant effect was greater than over the interval of lengths where the shortening was performed in myogram *b* (cf. length dependence of movement effect section 5)

Fig. 5 B shows that prestretch of the fibre did not affect the depressant effect of shortening. The two myograms *b* and were recorded at ⌞.02 μm sarcomere length In both cases after allowing the fibre to shorten from 2.22 μm during the first cycle *In* myogram the shortening was preceded by a stretch from ⌞.0 to 2 ⌞ μm sarcomere length As can be seen the time course of force development during cycles –6 was quite sim lar in the two runs

5 *The length dependenc of the mov ement effect*

The depressant effect of act ve shortening was studied at various sarcomere lengths in the range 1 7 2.9 μm The experimental protocol was similar to that described in section 1 In the present experiments however the test and control contractions were initiated at d fferent sarcomere lengths to allow the fibre to redevelop force at the same length after the small (control) and the large (test) release The shortening was performed during the last cycle of a partially fused tetanus The amount of shortening was 0 29–0.31 μm/sarcomere during the test and 0 05–0 08 μm/sarcomere during the control

The results from 3 expts. are summarized in Fig. 6 The depressant effect has been expressed in per cent of the redeveloped force in the cont ol run at each length. It can be seen that for a gi en amplitude of shortening the force depression was maximum when the shortening ended at 2 1–2.2 μm sarcomere length. The depressant effect decreased when the shortening was performed at longer and shorter lengths. The mechanical behaviour of a given fibre was consistent during repeated contractions at various sarcomere lengths. It is evident from Fig. 6 however that the length dependence of the movement effect exhibits a somewhat greater variability from fibre to fibre than observed for the relationship between tetanic force and sarcomere length (see Fig. 1 Edman 1966 and Fig. 3 Gordon Huxley & Julian 1966)

6 *The effect of shortening on th force–velocity relationship*

The following experiments were performed to find out if the depression of isometric force that occurs in response to active shortening is paralleled by a decrease of the maximum velocity of shortening. The same general approach was used to study the depressant effect as described in Fig 1 A i.e the redevelopment of force was recorded after the fibre had been allowed to shorten by a small and a large amount during the activity period The velocity of unloaded shortening (V_0) was determined during the rising phase of the redeveloped twitch using the multiple-release method described previously (Edman 1979) The results of 1 expt are illustrated

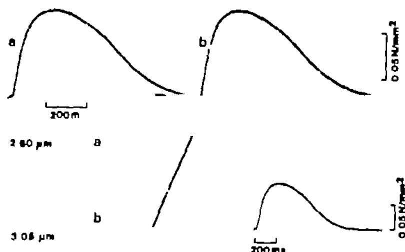


Fig 4 Two isometric twitches recorded with a time difference of one minute Record *a* no preceding shortening Record *b* contraction preceded by passive shortening The higher base line (resting tension) under myogram *a* refers to the situation prior to release in myogram *b* Inset recordings in *a* and *b* repeated and superimposed upon each other on oscilloscope screen Temperature 1 9°C Cross-sectional area 5 604×10 mm

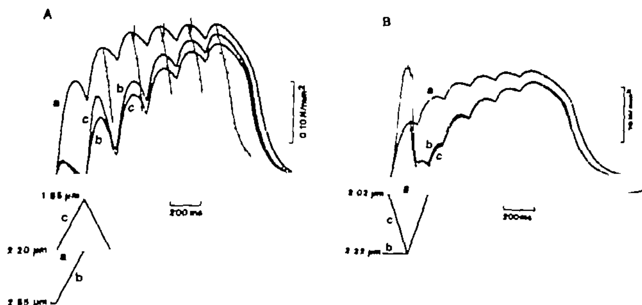


Fig 5 (A) Isometric force development at 2.20 µm sarcomere length during incompletely fused tetanus in the absence (myogram *a*) and in the presence of shortening (myogram *b*) or shortening restretch (myogram) during the first two cycles Filled circles (connected by thin line) indicate peak redevelopment of tension at 2 20 µm sarcomere length after a small (0 09 µm/sarcomere) release carried out at different times during cycles 2–6 in the absence of shortening or shortening restretch during cycles 1–2 Arrows indicate tension values used as control for the peak twitch force in cycles 3–5 of myograms *b* and Lower records (retracings) position of puller arm indicating sarcomere length during myograms *a–c* Temperature 1 6°C Cross-sectional area 13 914×10 mm² (B) Force development at 2.02 µm sarcomere length of incompletely fused tetanus in the absence (myogram *a*) and in the presence of shortening (myogram *b*) and stretch-shortening (myogram *c*) during the first two cycles. The three myograms have been superimposed photographically Temperature 1.3°C Cross sectional area 9 791×10⁻ mm²

ble 2 *The elocity of unloaded shortening (V₀) measured during single twitch after depression of the etric force by active movement*

	Temp °C	V of control twitch (L/sec)	V of depressed twitch (% control)	Peak force of depressed twitch (% control)
xs 1978	1 1	2.32	100	84
1979	0 7	2 19	96	58
1979	0 4	2.19	97	68
1979	1 4	2.16	103	50
			99±	65±7

ean ± S.E. of mean

e movement effect. The Q_{10} based on 3 expts. as 0 95±0 16 (S D)

ISCUSSION

aracteristics of the movement effect

he present study has provided further evidence at active shortening involves some change in the ontractile system that reduces the fibre abiity to roduce force over a relatively long time (800–900 s) after the movement. The results have shown at this depressant effect appears during a fused etanus as well as during a twitch or a partially sed tetanus It can furthermore be concluded that e phenomenon is related to a *shortening* move ment only (cf Edman Elzinga & Noble 1978) a stretch performed immediately before or after the hortening (at sarcomere lengths <2.25 μm), was ound to have no significant influence on the de ressant effect. Deactivation by shortening has lso been shown to occur in amphibian slow muscle ibres (Lännergren 1978) mammalian skeletal muscle (Joyce & Rack 1969 Joyce et al 1969) and amphibian and mammalian myocardium (Brady 1966 Edman & Nilsson 1971 Bozler 1972, Kaufmann, Bayer & Harnasch 1972, Bodem & Sonnenblick 1975) i.e the phenomenon would seem to apply for striated muscle in general This finding is fully in line with the view (see below) that the movement effect is a specific consequence of the sliding-filament process under conditions normally xisting in the living fibre

There is evidence that the magnitude of force depression after shortening is dependent on the state of activation of the fibre when the shortening occurs. The decrease in isometric force for a given amplitude of shortening was thus found to be smaller during a tetanus than during a twitch The effect was also reduced after treating the fibre with caffeine which can be presumed to increase the concentration of calcium in the myofibrillar space (Ebashi 1976 Blinks et al. 1978). These observations accord with the previous finding (Fig. 4 Edman 1975) that the depressant effect is steadily increased as the shortening is carried out at later times during a twitch when the activity decays

The movement effect described here should be distinguished from the decrease in contractile strength that occurs when an excessive amount of work is produced during contraction. An increase of the work performance above approximately 2 μJ/cm² (Edman & Mattiazzi unpublished results) causes a decrease of the tetanic force which persists during continued (4–6 s) stimulation of the fibre (Abbott & Aubert 1952 Edman 1966 Maréchal & Plaghki 1979) This force deficit increases with the amount of work produced. By contrast, the movement effect described in the present paper is not significantly dependent on the force or the work output during the shortening phase (Fig. 6 Edman 1975). Furthermore full contractile strength is regained during continued isometric activity These features of the movement effect suggest that the transitory decrease in mechanical performance that is produced by shortening is not due to exhaustion of chemical energy for the contractile process. Evidence obtained in studies of ATP and creatine phosphate breakdown during unloaded shortening supports this view (Abbott & Howarth 1973 Curtin & Woledge 1978, Homsher & Kean 1978)

Curtin & Woledge (1978) direct attention to the possibility that the movement effect might bear some relation to the shortening heat i.e the extra

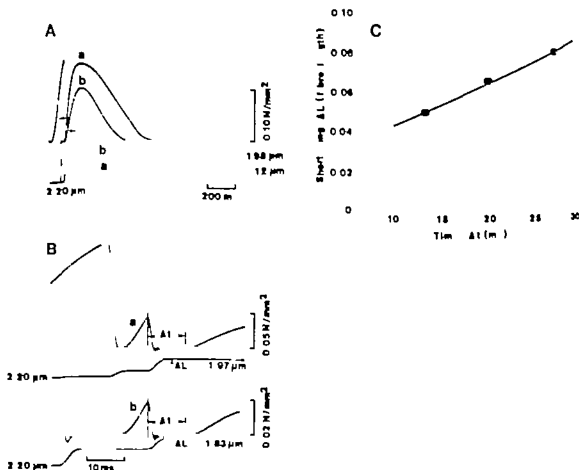


Fig 7 Measurement of force and unloaded velocity of hortening (V) after active movement during single (A) Redevelopment of tension after small (0 08 µm/sarcomere myogram *a*) and large (0.2. µm/sarcomere m *b*) movement. (B) Release recordings for determination of V carried out after small (myogram *a*) and large (m movement at times indicated by arrows in A ΔL amplitude of release in V analysis. Δt time from onset of r to beginning of force redevelopment Note different tension scales for myograms *a* and *b* (C) Relationship betw ΔL and Δt in control twitch (●) and in twitch depressed by large movement (O) Each point is the mean of three sep recordings The slope of the line relating ΔL to Δt (regression analysis) is 2 19 and 2 12 *L*/sec in control and test resp tively (regression coefficients 0 996 and 0 989 $n=9$) Temperature 0 4°C Cross sectional area. .5 659×10⁻ mm²

in Fig 7 In this expt the large movement depressed the peak isometric force to 58% of the control value (Fig 7A) Examples of release recordings used for determination of V after the small and the large movement are shown in Fig 7B As can be seen the time (Δt) from the onset of release to the onset of force redevelopment was very nearly the same in the two cases The complete analysis of V_o, based on three different amplitudes of release (ΔL) is shown in Fig 7C V_o, the *slope* of the line relating Δt to ΔL was not significantly different in the depressed twitch and the control twitch The results of four experiments including that illustrated in Fig 7 are summarized in Table ? It can be seen that whereas the force was depressed to 50–84% by active shortening in the different expts V remained unaffected to within 4% of control

7 The influence of temperature on the movement effect

In order to determine the temperature dependence of the movement effect the *speed* of force redevelopment (*dP/dt*) after shortening was studied during fused tetani at 1.50–2 00 and 11 25–11 38°C By the approach illustrated in Fig 2 (inset) it was possible to compare the movement effect at two different temperatures at maximum degree of activation of the fibre For determination of the depressant effect *dP/dt* was measured in both test and control at 50% of maximum tetanic force A rise in temperature did not cause any significant change

[illegible]tde of the movement effect, with a maximum [illegible]r 2.1 μm sarcomere length does not reflect the [illegible]gree of activation of the contractile system at [illegible]rious sarcomere lengths. Results reported by [illegible]nks et al (1978) suggest that the amount of cal[illegible]um released by the action potential is maximum [illegible]tween 2.4 and 3.0 μm sarcomere length in dif[illegible]rent fibres and that the calcium release is steadily [illegible]duced at shorter and more extended lengths. The [illegible]ngth dependence of the depressant effect would [illegible]us seem to be governed by a factor that over[illegible]wers the influence of activation.

The interesting possibility emerges from these [illegible] sults that the relative position of the A and I fila[illegible]ents during the movement somehow determines [illegible]e magnitude of the depressant effect. It is evident [illegible]om Fig. 6 that the depression of force does not [illegible]ange in proportion to the control isometric ten[illegible]on as the overlap area between the thick and thin [illegible]aments is altered. The results could mean that the [illegible]yosin bridges are progressively effective in deacti[illegible]ting the contractile system the closer to the [illegible]ntre of the thick filament the bridges are located [illegible] this case the movement effect would rise to a [illegible]aximum as the sarcomere spacing is reduced to [illegible] 1 μm. The effect would decline below this point [illegible] I filaments intruding from the opposite half of the [illegible]rcomere would prevent the centrally located [illegible]ridges from interacting with the same side's I fila[illegible]ents.

[illegible]his work was supported by grants from the Swedish [illegible]edical Research Council (project no. 14X 184) and the [illegible]uscular Dystrophy Association of America.

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heat produced during active shortening and that is not explained in terms of ATP splitting or the creatine kinase reaction. The heat of shortening and the movement effect are both largely independent of the speed of shortening; they increase with the amplitude of shortening and they both reach a maximum at the plateau of the length-tension diagram (cf. Curtin & Woledge 1978). Other observations, however, make it seem unlikely that the two phenomena are causally related. Thus the heat of shortening has been found to decline progressively when contractions with shortening are repeated at 5 sec intervals (Dickinson & Woledge 1974). No such dependence on the previous activity of the muscle applies for the force depression after shortening.

The movement effect does not include a change of V_0, the measured velocity of shortening at zero load. This accords with the observation that the speed of shortening against a small load ($P<0.01$ P_0) remains very nearly constant as the fibre shortens between 2.4 and 1.7 μm sarcomere length under load-clamp conditions (K. A. P. Edman, unpublished data). The constancy of V_0 also agrees with the previous finding (Edman 1979) that V_0 is independent of the fibre's capacity to produce force within the range P_0–0.1 P_0. The lack of effect on V_0 would seem to rule out that shortening produces a passive resistive force that accounts for the decrease in contractile strength after the movement. An increase of the 'intrinsic' load would cause a relatively large decline of V_0 considering the steep relationship between force and velocity of shortening at loads close to zero.

Nature of movement effect

Deactivation. The relatively long duration of the movement effect (nearly one second) suggests that this phenomenon is distinct from the mechanical transients that occur after a quick length change (stretch or release) of an active muscle fibre (Podolsky 1960, Huxley & Simmons 1971). The mechanical transients, which involve changes of both tension and shortening velocity, can be explained in terms of the kinetic properties of the cross-bridges (Civan & Podolsky 1966, Huxley & Simmons 1971, Bressler & Clinch 1974, Ford, Huxley & Simmons 1977) and are not likely to reflect an altered state of activation of the contractile system. The movement effect, on the other hand, has features that are characteristic of deactivation (Edman 1979), i.e. the force output is reduced without change of V_0.

In explaining the deactivating effect of shortening the following mechanisms may be considered:

1. Increased rate of sequestration of activator calcium by the sarcoplasmic reticulum.

2. A structural change in the contractile system that leads to a reduced actin–myosin interaction.

The first alternative appears unlikely in the light of the present results. Such a mechanism would require that shortening increases the effectiveness of the calcium pump of the sarcoplasmic reticulum and that this effect persists over several hundreds of milliseconds after the movement. In such a case a passive shortening performed immediately before stimulation would be expected to affect the subsequent course of contraction. The results have shown, however, that the shortening has to occur during activity in order to reduce the contractile strength.

In considering the second alternative, the low temperature dependence of the movement effect, with a Q_{10} close to 1, is consistent with a change of a protein structure. The precise nature and location of the structural change in the myofilament system cannot yet by defined, however. It has previously been suggested (Edman 1975) that active shortening causes some change of the regulatory protein complex of the I filament that leads to a transitory impairment of the actin–myosin interaction. The structural change may decrease the affinity of the binding sites for calcium and thus reduce the amount of activator calcium in the contractile system (cf. Edman & Kiessling 1971 and Kaufmann et al. 1972). Another possibility worth taking into account is that shortening produces a structural change, or dislocation, of a fraction of the myosin bridges thereby decreasing the ability of these bridges to interact with the actin filament.

The idea that shortening reduces the concentration of calcium in the myofilament system is attractive as it conforms with the finding that the movement effect is less pronounced at a higher degree of activation of the fibre, i.e. when the concentration of calcium is increased. The fact that shortening causes some abbreviation of the mechanical activity (Fig. 1) is also in line with the hypothesis that calcium is mobilized from the contractile system during the shortening phase.

The length dependence of movement effect. There is reason to believe that the length dependence

ence of the movement effect, with a maximum near 1 μm sarcomere length does not reflect the degree of activation of the contractile system at various sarcomere lengths Results reported by Blinks et al (1978) suggest that the amount of calcium released by the action potential is maximum between 2.4 and 3.0 μm sarcomere length in different fibres and that the calcium release is steadily reduced at shorter and more extended lengths The length dependence of the depressant effect would thus seem to be governed by a factor that over powers the influence of activation.

The interesting possibility emerges from these results that the relative position of the A and I filaments during the movement somehow determines the magnitude of the depressant effect. It is evident from Fig 6 that the depression of force does not change in proportion to the control isometric tension as the overlap area between the thick and thin filaments is altered. The results could mean that the myosin bridges are progressively effective in deactivating the contractile system the closer to the centre of the thick filament the bridges are located In this case the movement effect would rise to a maximum as the sarcomere spacing is reduced to 2 1 μm The effect would decline below this point as I filaments intruding from the opposite half of the sarcomere would prevent the centrally located bridges from interacting with the same side s I filaments

This work was supported by grants from the Swedish Medical Research Council (project no 14X 184) and the Muscular Dystrophy Association of America.

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Topical application of penicillin into hippocampal in vitro slices. A methodological study using benzyl (^{14}C) penicillin

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OLOFSSON, S., RIDDERHEIM, P.-Å. & SILFVENIUS, H. Topical application of penicillin into hippocampal in vitro slices: A methodological study using benzyl (^{14}C) penicillin. Acta Physiol Scand 1980, 109: 27–36. Received 26 July 1979. ISSN 0001-6772. Departments of Physiology and Neurosurgery, University of Umeå, Sweden.

A gas pressure system is employed for topical application of pico/femtolitre volumes of 9 mmol/l benzyl (^{14}C) penicillin into guinea-pig in vitro transverse hippocampal slices. Control of pressure pulse parameters enables ejectates, computed after liquid scintillation, from 10 µm, 5 µm and 2 µm pipettes with high reliability of ejection. A detailed study was done on the ejection performance of 5 µm and 2 µm pipettes. It shows that for the 5 µm tips with the chosen ejection parameters, feeding pressure = 0.5 MPa, feeding pulse duration 90 ms, a 99% ejection incidence was obtained (200). The mean volumes ejected were 0.12 and 0.5 nl (= 30) for one and four pulses delivered respectively. The corresponding ejectate means obtained from five 2 µm pipettes given a feeding pressure of 1.5 MPa, and a feeding pulse duration of 70 ms, were 0.04 and 0.13 pl. Statistical evaluation of the individual pipette ejection performances gave characteristic regression slopes within each pipette group. A comparison between visually controlled injection into oil and injection into slice showed close correspondence. The applicability of the ejection method is discussed in relation to functional studies on the conversion of single neurones into epileptic ones.

Key words: Benzylpenicillin, radioactive, pressure microinjection, in vitro hippocampal slice

Benzylpenicillin has since long been used as an epileptogenic agent in experimental studies. Topical application of the substance onto single cells may be made from micro pipettes using either mechanical, iontophoretic or thermal methods. In studies on the effect of benzylpenicillin on single neurones, a quantification of the topically applied substance is desirable since functional alterations evoked should preferably be correlated to the ejectate volume as well as to the exact site of application. A reasonably accurate method of quantification of small amounts of ejected benzylpenicillin is therefore required. Such a method could lead to the determination of the neuronal membrane region contacted by the ejected substance. Applied extracellularly to neocortex, benzylpenicillin alters the neuronal discharge pattern in the incomplex (eg. native circuit towards excitation (Walsh 1971, Curtis et al. 1972). The present report describes a method of pressure extrusion of benzylpenicillin from glass micro pipettes. It displays, as will be shown, a high degree of reliability, showing only moderate scatter between the individual ejectates as evidenced by the amounts of ^{14}C-benzylpenicillin, injected into guinea-pig transverse hippocampal in vitro slices. The method will therefore later be applied to study the effect of benzylpenicillin on single hippocampal cells.

METHODS

Transverse hippocampal slices, 400 µm thick, were cut from guinea-pigs weighing 300–500 g, anesthetized with chloroform, in a box into which a 95% O_2–5% CO_2 gas mixture was led at 4 l/min. The slices were maintained in vitro according to the method of Skrede & Westgaard (1971). A stock solution of µCi/µl benzyl (^{14}C) penicillin (S.A. 55 mCi/mmol, 2.0 GBq/mmol, Amersham, England) was prepared with absolute ethanol. A certain amount of the solution was further diluted 1:3, while the rest was evaporated under N_2 gas and stored at −20°C. Eleven glass tubes were pipetted with 10 µl of the 1:3 dilution. One

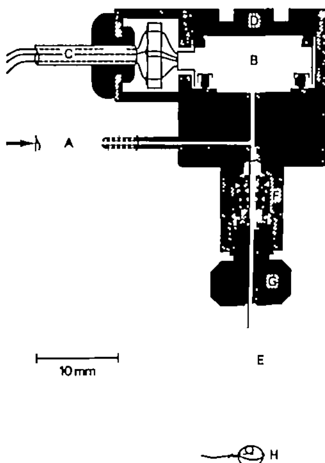


Fig 1 Schematic side view of the Aluminium pipette holder used for pressure ejections of ¹⁴C-penicillin *A* Impolen tubing connected to the magnetic valve arrow indicating feeding pressure wave inflow *B* Miniature pressure transducer measuring pulse waves see Fig 3 A and 4 A connected via *C* to amplifier system and screwed into holder by screw *D* *E* ¹⁴C-penicillin glass pipette *F* Inset piece to minimize angling of pipette *G* Screw cap for pipette with O-rings. *H* Oil droplet in silver loop and a penicillin spherule

of them was further diluted ad 1 000 μl Of this dilute 10 μl was transferred to a scintillation vial which radioactivity was determined This served as reference ×1 000 for the other 10 glasstubes pipetted They were evaporated under N and stored until the day of expt

10 μl of filtered hippocampal slice bath medium was then added to a tube used in one expt solving the ¹⁴C penicillin. From the 10 μl solution a small amount was sucked into a thin glass capillary drawn over heat by hand from a 2 mm diameter glasstube This capillary was inserted into the shaft region of a GC 1?0F glass pipette (Clark Electromedical Instrs England) drawn to a tip that was broken to a particular size ~10 μm A ¹⁴C-penicillin pillar about 500 μm above shaft level was usually kept in the pipettes All air bubbles were allowed to escape from the pipette solution

The ¹⁴C-penicillin concentration was 9 mmol/l The ¹⁴C-penicillinpipette was then thightly screwed into it Al-holder of the pressure injection system. This consisted of (cf Fig. 1): *a*) a pressure source with regulator connected with an Aluminium embedded tubing (Delair 1300" Samuel & Moore USA) to *b*) a magnetic [illegible] way valve (Lucifer Switzerland) of 9 4 or 2.5 MPa nominal capacity and with a response time of 15 ms of 90 feeding pressure The valve was driven by *c*) a pulse generator with stepless duration manoeuvering and [illegible] connected to the *d*) pipette holder with a polypropylene tubing (Impolen 22-PP ITE USA) The holder enclosured *e*) a miniature pressure transducer either type AF 830 D or type AF 831 F(AS Mikro Elektronikk Norway) with ranges of 0–1 and 0–5 MPa being in air contact with the top of the ¹⁴C-penicillin fluid pillar The output from the transducer was fed via an *f*) amplifier system to one beam on the CRO Pressure pulses recorded by the transducer are shown in Figs 3 A and 4 A The peak pressure P_p, and feeding pressure P will in the following be referred to

The ¹⁴C-penicillin pipette and its holder was carried by micromanipulator driven by a stepmotor Under visual microscopic control the pipette was advanced in the slice chamber until its tip mirrored on a slice surface It was then further advanced at an angle of 10 degrees to the vertical plane to a depth of 150 μm in the stratum radiatum of the CA hippocampal region The ejection was done at this depth

Except in early expts identical pressure pulses were given 1× 2× 3× and 4× and a series of ten slices were injected at each pressure group The reliability and ejection scatter of the method could thereby be assessed

Between slice ejections the pipette was cleansed in the moist gas of the chamber and the ¹⁴C-penicillin droplet extruded was sucked off with the wet tip of a tissue softpaper roll The possible remaining contamination of the wiped tip never resulted in above background detectable counts even if the tip was kept in a slice but giving no pressure pulse for periods more than 5 min

In the course of the experiments the ejection method was modified Prior to injecting into a slice an ejection was made into a tiny oil droplet kept in a 300 μm diameter thin silver wire loop The ¹⁴C-penicillin spherule ejected into oil could clearly be visualized in the viewing microscope if of a diametre >10–15 μm Usually the P was adjusted so that the ¹⁴C-penicillin spherule in oil had diameters of 80–100 μm corresponding to 0 014–0 [illegible] nl The pressure adjustments were made for each pipette

The tip sizes used were between and 10 μm The tip size was checked after the expt and found unchanged Blow outs were seen in slices leaving tiny temporary craters if large pipette tips were used together with high pressure pulses i e 10 μm tips P around 0 5 MPa After injection the pipette was withdrawn and the injected slice was removed from the bath with metal spatula into a scintillation vial containing 1 ml of tissue solubilizer (Soluene 100 Packard USA) The injected slices were allowed to disintegrated at +40°C for 30 min after which ml of scintillation liquid was added to each vial

The vials were counted after equilibration in Liquid Scintillation Spectrometer (Packard 3310) for 5 min [illegible] Occasional series were counted 5 min 10 or 20 to assess scintillation variability Counts were converted [illegible] to volumes expressed as pl or nl

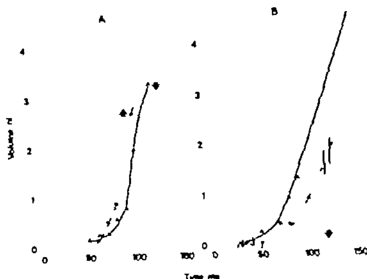


Fig. 2 A. Response curves of 10 µm pipette from which ^{14}C-penicillin was ejected at feeding pressure, P of 49 kPa during increasing (●—●) and decreasing (▲—▲) pulse lengths. Each nl amount refers to the ejectate in one slice. *2 B.* Pressure—response curve of a 10 µm pipette (▲—▲) through which 59 kPa pulses of 50–150 ms durations were given, being compared with that of 5 µm pipette (●—●) tested on similar pressure parameters. The latter one was blocked at pulse duration 120 ms. Each value refers to ^{14}C-penicillin ejectates from one slice. Free hand fitting of curves.

Quenching as checked by comparing counts obtained on vials containing Soluene 100 and scintillation liquid ity and such into which non-injected slices or the tiny iver thread loops had been dropped. Differences above ackground values were not obtained.

Statistical evaluation and plotting was done using TATPAC for NORD-10 computer programmed at the Karolinska Institutet, Stockholm.

RESULTS

The quantification of pressure ejected ^{14}C-penicillin rom glass macro pipettes into the CA stratum radiatum of guinea-pig hippocampal in vitro slices as made using a number of pipettes with tip sizes 5 and 10 µm. Initially mainly single injections were made during which the feeding pressure P was kept constant while driving pulse, duration T as altered. Low P injections were made with 5 and 10 µm tips.

The lowest P tested on 10 µm pipettes was 29 kPa. With T pulses of 50–100 ms durations, above background detectable radioactivity was found but almost every other slice contained no detectable radioactivity. When the T s were increased to 120–150 ms ejectates between 0.16–1.1 nl were obtained. An increase in P_f to 49 kPa gave a more reliable dose-response curve for 10 µm pipette as is shown in Fig. 2 A.

For this particular 10 µm pipette the P pulses were first given in an increasing T duration sequence (●– – ●) and then in the opposite direction (▲—▲). As seen in Fig. 2 A both sequences gave similar slopes of the curves with different ejectates at the individual pulse duration steps. In order to obtain more data on the relation between P_f and T at low P values nine 10 µm pipettes were tested at the P levels 29, 49 and 59 kPa, using T s between 50–150 ms. The individual ^{14}C-penicillin ejectates varied somewhat as is seen in Table 1 A.

With a P of 29 kPa and with T_d pulses between 50–150 ms of the twenty-six injections given ten slices contained extremely small amounts (0.01–0.02 nl) suggesting that in 39% there was complete or partial blocking of the pipettes.

In any of the three pipettes tested at this P the small ejectates could occur at any T duration. Thus despite variations in volume there was a positive correlation between T_d-s and nl in each pressure group. Increasing the P to 49 kPa gave a more reliable outcome of ^{14}C-penicillin ejectates from the three 10 µm pipettes tested. In this injection series there were no borderline ejectates only three

Table 1 *Amounts of ¹⁴C penicillin nl in hippocampal slices obtained with low P_f s at varying driving pulse durations*

Driving pulse duration		50	60	70	80	90	100	110	150 ms
A *10 μm pipettes*									
I 29 kPa	1	0.01	0.37	0.01	0.38	0.[illegible]8	0.0	0.01	0.01
		0.16	0.14	0.0[illegible]	0.01	0.11	1.07	1.61	[illegible].99
	3	0.0[illegible]	0.06	0.03	0.0	0.09	1.[illegible]5	1.94	1.9
Mean		0.07	0.19	0.02	0.14	0.16	0.78	1.18	1.31
II 49 kPa	4	0.17	0.[illegible]6	0.59	0.79	1.97	5.[illegible]	3.28	4.10
	5	0.11	0.1	0.04	0.03	0.70	1.1	[illegible]63	0.06
	6	0.15	0.19	0.33	0.5[illegible]	1.15	[illegible].33	[illegible]08	[illegible].31
Mean		0.14	0.19	0.32	0.45	1.27	[illegible].89	[illegible].87	3.16
III 59 kPa	5	0.19	0.3	0.7[illegible]	0.88	1.71	3.67	[illegible].99	4.51
	6	0.46	0.54	0.64	1.10	1.66	3.38	3.91	[illegible].46
	7	0.[illegible]5	0.16	0.41	0.96	1.31	2.81	[illegible].58	4.[illegible]
Mean		0.30	0.34	0.59	0.98	1.56	3.[illegible]9	3.49	4.83
B *5 μm pipettes*									
I 59 kPa	1	0.15	0.05	0.08	0.4	1.1	0.9	0.00	3.[illegible]3
	2	0.07	0.00	0.14	0.12	0.16	0.30	0.69	0.88
II 78 kPa	3	0.07	0.11	0.12	0.14	0.40	0.73	0.75	0.68
	4	0.04	0.11	0.00	0.[illegible]1	0.34	0.56	0.90	0.93
III 98 kPa	5	0.14	0.19	0.45	0.66	1.16	1.71	1.60	1.20
	6	0.06	0.11	0.10	0.19	0.[illegible]5	0.48	0.54	0.43

slices contained ejectates distinctly lower than the other (i e 0 03–0 06 nl) They were all obtained from one pipette The largest ejectates obtained at P_f 49 kPa with pulse duration extremes of 50 and 150 ms were 0 17–5 31 nl Elevating the P_f further to 59 kPa increased the repeatability of injections the small ¹⁴C penicillin amounts lacking in this series of three 10 μm pipettes The largest ejectate volumes obtained at this P_f level with pulse extremes of 50 and 150 ms were 0 46 and 5 46 nl Expectedly least interpipette scatter between individual ejectates was obtained at this P_f

Thus low pressure injection of ¹⁴C penicillin with the present system into hippocampal slices was obtained with acceptable but somewhat variable yields provided the P_f is of the order of 50–60 kPa and the driving pulse duration preferably exceeds 50 ms Clogging of the tip occurred often if lower P_f-s were used

In order to obtain some piece of information on the ejection performance of 10 μm vs 5 μm pipettes a P_f of 59 kPa was fed into two pipettes of either size The outcome of this comparison is shown in Fig 2B T_d s of the order of 30–60 ms gave practically no detectable amounts of ¹⁴C-penicillin in the two slices Pulses 70 ms or longer yielded ejectates which except for one clogging incidence increased similarly but of different volumes In this particular experiment the 5 μm pipette was further kept in an additional slice for 25 min with no pressure pulse being given It was found that 0 06 nl of ¹⁴C penicillin had diffused out from the tip during that period

Ejectates obtained from six 5 μm pipettes are further listed in Table 1 B The lowest P_f tested, 59 kPa, was equal to the highest tested P_f on 10 μm pipettes Comparing the 5 μm and 10 μm pipettes, it was found that the general yield at this P_f is comparable to P_f s for the 10 μm pipettes of about 30 kPa Of the nineteen injections given at P_f 59 kPa measurable amounts for the two 5 μm electrodes were obtained in sixteen i e about 0% of presumed clogging occurred (cf Table 1 B I) Increasing the P to 78 kPa the reliability of ejectates increased together with a decrease in interpipette scatter (cf Table 1 B II) A P given to a 5 μm pipette identical in duration but twice of that delivered to a 10 μm pipette gave thus on the whole comparable ¹⁴C-penicillin ejectates (compare Table 1 A II with 1 B III)

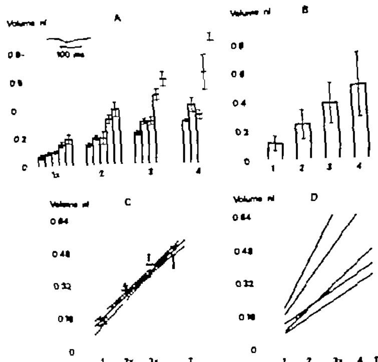


Fig 3 *A* Histogram showing means and SD.s of five 5 μm pipettes from which ^{14}C-penicillin was ejected Each column corresponds to ten slices. The individual pipettes were pressure ejected 1–4 with identical pulses, the feeding pulse being 70 ms and 0.50 MPa The actual pulse pressure length was 150 ms the risetime 28 ms. The peak pressure P_{pe} was 0.20 MPa. Undetectable amounts were found in 2 out of 200 slices. Inset shows pressure pulse recorded by transducer as described in Fig. 1*B* From 3 A, pooled ^{14}C-penicillin ejectates. Means, and SD:s are shown. Dots at abscissa in A and B refer to "zeros" found at one and two pulse injections. The zeros are included in calculations. *C* Linear regression plot for individual volumes of ^{14}C-penicillin ejectates obtained from one 5 μm pipette, graphically represented by the third column in each injection group in Fig. 3 A A 95% confidence interval for mean of volume is drawn. *D* Linear regression slopes of fifty volumes obtained at each ejected pressure number group from five 5 μm pipettes

e ^{14}C-penicillin ejectates from 5 μm pipettes with v pressure ejections (60–100 kPa) yielded an out me in 95% but with a high scatter between the ividual amounts A doubling of the *P* (120–200 a) for 5 μm pipettes should therefore be per rmed if ejectates are to be obtained comparable those from 10 μm pipettes. In order to ascertain a ther accuracy in the test of the reliability of any 5 m pipette ejection performance the experimental ocedure was modified in the following way Prior injecting into a slice a chosen P and T were set obtain a ^{14}C-penicillin spherule in oil as described METHODS As the viewing microscope allowed accuracy of the 70 μm diameter magnitude of the -penicillin spherule in oil, the P and T were set to produce a ^{14}C-penicillin spherule of about 80–100 μm diameter corresponding to about 0.5 nl Once the pressure pulse parameters were set for each individual 5 μm pipette they were left unaltered The only parameter being changed was the number of pulses given Depending on the ejection characteristics of the individual 5 μm pipettes the size of the obtained ^{14}C-penicillin spherule in oil varied between the pipettes. The reliability of the individual pipettes was then tested by giving identical pulses 1× 2× 3× and 4× to ten different slices in each pulse repetition group Forty slice ejectates were thus obtained from each pipette

The outcome of the test performance on five different 5 μm pipettes with a P of 0.50 MPa and P of

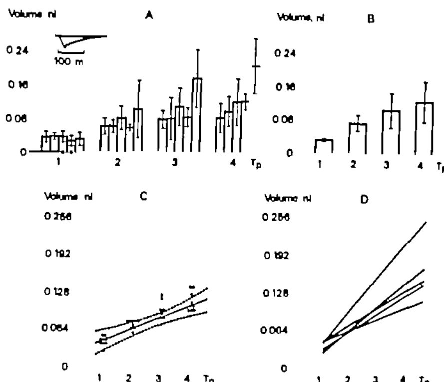


Fig 4 A Quantities of ¹⁴C-penicillin encountered in ?00 slices injected with a P of 1 5 MPa from five 2 µm pipettes with 1–4× pressure pulses of 70 ms Pressure pulse wave measured at top of pipette shown in inset The size of the test-drop of ¹⁴C-penicillin in oil was 30–40 µm in diameter corresponding to about 30 pl The peak pressure wa 0 45 MPa Means and SD:s are shown Each column refers to ten slices Symbol at abscissa in two first group columns refer to undetectable amounts in four slices *B* Histograms from 4 A with means and SD s *C* Linear regression plot from one 2 µm pipette represented by the fourth columns in 4 A showing distribution of volumes and 95% confidence interval for mean of volume *D* Linear regression slopes of fifty volumes obtained at each pressure number groups ejected from five µm pipettes

0 ?0 MPa and a driving pulse of 70 ms on totally ?00 hippocampal slices fifty slices in each pressure group is shown in Fig. 3 A

The histograms of Fig. 3 A indicate that the used pressure parameters on 5 µm pipettes secure a high repeatability in ejection performance clogging of the tip occurring only in 1% if one or two pressure pulses are delivered Fig 3 A further illustrates that the individual pipettes have their own characteristic ejectate slopes related to delivered pulses If the five injection series are pooled together the Means and SD:s obtained from injections into fifty slices at each number of pulses are shown in Fig 3 *B* From these data it can be concluded that one pressure pulse secures in 99% of the slice injections an average volume of 0 1? nl ¹⁴C-penicillin

Data on the distribution of individual ¹⁴C-penicillin ejectates from a single 5 µm pipette are shown with a linear regression plot and a 95% confidence interval for mean volume in Fig 3 C This plot was made on a single pipette whose performance is represented by the third column at each injection leve in Fig 3 A The linear regression slopes of the fiv 5 µm pipettes are shown in Fig 3 D

In summary a feeding pressure of 0 50 MPa corresponding to a peak pulse pressure of 0 ? MPa delivered by a driving pulse of 50 ms secure a 99% ejectate yield from 5 µm pipettes with mea ¹⁴C-penicillin volumes between 0 1?–0 5 nl depend ent on whether 1 or 4 pulses are delivered

Pipettes with tip sizes 2–2 5 µm here referred t as ? µm were tested similarly as were the 5 µ pipettes The ? µm pipettes would expectedly re quire higher pressure pulses to ascertain high relia bility in ejectates but occasionally low P_f s wer tested The T_d pulse duration was however i such an instance increased to 100 ms The P_f s ll ?9 49 and 196 kPa were tested with single pressu pulses and gave the following ¹⁴C penicillin mea amounts (*n*=5) 0 11 0 0.24 and 0 85 nl Th longer pulse duration with the ? µm pipette th secured ejectates of similar magnitudes as thos

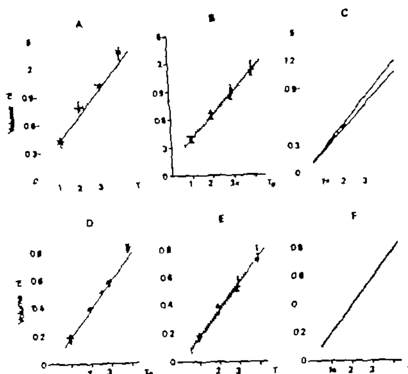


Fig. 5. Upper diagrams show linear regression slopes of ^{14}C-penicillin ejectates from a 5 µm pipette delivered into ten slices (*A*) and into ten oil droplet (*B*) at each pressure pulse group. The injections were given alternatingly into the two media, with cleansing pulse inbetween. Distribution, 95% confidence interval for mean of volume and linear regression lines are given. *C*. Comparison of linear regression slopes for A and B. Lower diagrams illustrate linear regression plots of ^{14}C-penicillin ejectates from a 2 µm pipette delivered into ten slices (*D*) and into ten oil droplets (*E*). The injections were delivered as described above. Distribution, 95% confidence interval for mean of volume and linear regression lines are given and compared, *F*.

obtained with half the pulse duration, from 5 µm pipettes.

Five µm pipettes were tested in the same manner as the 5 µm pipettes. The size of the ^{14}C-penicillin spherule obtained in oil was kept around 30 µm diameter for each pipette. The spherule size was achieved with a P of 1.5 MPa, corresponding to a P of 0.45 MPa and driving pulse of 70 ms (see inset Fig. 4 A).

As shown by the histogram in Fig. 4 A, the patency of the µm pipettes in 200 slices was maintained in 98% of the injections. In the pulse number group blocking occurred in 4 out of 50 single injections while in pulse number groups 2–4× no blocking occurred. The blocking incidence was thus 1% larger for the 2 µm than for the 5 µm pipettes tested. Data on the distribution of the individual ejectates obtained from one pipette are shown in Fig. 4 C showing the 95% confidence interval for mean of volume for the ten injections at each pressure group and the linear regression of the ^{14}C-penicillin ejectates as a function of pulse magnitude. The mean volumes and SD.s are shown in Fig. 4 B for the five injection series pooled together. The linear regression data of the ^{14}C-penicillin ejectates obtained from the five µm pipettes are shown in Fig. 4 D. From these it may be seen that a single MPa pressure pulse of 70 ms duration on the average yields a 0.035 nl volume.

Comparing the µm and 5 µm pipettes Figs. 3 and 4 clearly illustrate the difference in volume magnitude between the two pipette samples chosen.

In conclusion high reliability is gained with ^{14}C penicillin ejectates from 2 µm pipettes with a feeding pressure of 1.5 MPa and a pulse duration of 70 ms. 2–4× pulses yield a 100% outcome, single pulse injections had a 9?% yield. The mean amounts obtained with the extreme pulse number groups (1×

and 4×) were of the order of 0 03 and 0 15 nl respectively corresponding to spherule diameters of 40–65 μm

When physiological experiments with ^{14}C-penicillin are pursued histological controls may be necessary to make In such instances scintillation of slices may not be done Determination of ^{14}C penicillin ejected into oil could therefore by inference be comparable to the volume injected into a slice from one and the same pipette used with identical injection parameters

In order to obtain information on the reliability of this approach experiments on the relation between ^{14}C-pencillin amounts injected into oil vs. slices were carried out in which the injections were done shifting the pipette from oil to slice A 5 μm and a 2 μm pipette was tested

The upper three diagrams of fig 5 show the results obtained from the comparison test performed with the 5 μm pipette The pressure parameters applied to the pipette were identical to those of the other five 5 μm pipettes investigated A illustrates the distribution of the individual volumes obtained with 1–4× pulses into the slice B the corresponding amounts injected alternatingly into oil The mean volume at each pressure pulse group in the two media are very similar differing from each other only at the 1/10 nl magnitude The 95% confidence interval for mean of volumes injected into the slice A is somewhat broader than that of the volumes in oil B indicating a higher tendency to partial blocking in the slice than in the homogenous oil

The slopes of the linear regression lines obtained from the slice and oil volumes are not identical however it can clearly be seen in Fig 5 C that from a practical point of view the difference is ignorable

The same slice vs oil injection test done on the 2 μm pipette gave similar results. They are shown in the lower sequence of diagrams in Fig 5 D and E illustrate the distribution of the individual volumes in slices and oil droplets the linear regression lines now almost identical F and the 95% confidence interval for mean volume being illustrated As for the 5 μm pipette the volumes vary somewhat more in slice than in oil but the means of each pressure level group are highly comparable differing from each other only at the 10–30 pl level

Injection into oil at different pressure pulse groups either from 5 μm or from μm pipettes thus provides a valuable check of the volumes injected into a slice Consequently visual estimation of the size of a non radioactive penicillin spherule injected into oil may serve as an estimate of a volume injected into a slice from a 5 μm or a μm pipette

DISCUSSION

Pressure microinjection is an alternative to iontophoretic and thermal methods Early pressure microinjection methods are described by Chambers & Kopac (1950) Methodological refinements have been done during the years but already in the early studies considerable exactness in ejectate quantities have been reported

A particularly favourable preparation to use in studies on microejection of substances considered crucial for nerve function was the giant axon preparation utilized by several authors shortly after the description of the injection method Grundfest et al (1954) Hodgkin & Keynes (1956) By combining electrolyte ejection with electrical recording from one and the same pipette Grundfest et al improved the functional spatial accuracy of the microinjection method

Further elaboration of the combined ejection-recording principle has later been performed by various authors and suggested as an alternative to the iontophoretic method Krnjević & Phillis (1963) Krnjević et al (1963) Woodward & Lindström (1977) McCaman et al (1977) Kålbström & Lindström (1978) By adding the use of radioactively labelled substances quantitative knowledge has been obtained of the effects of various substances on neuronal mechanisms Extra and intracellular pressure microinjection has thus been possible to make using either single or multibarrel pipette set-ups

Several studies have been performed with pressure ejection of substances and relating the ejectates quantitatively to the functional effects observed Studies on ionic mechanisms have thus been conducted (Chang et al 1974 Rose & Loewenstein 1976) or on transmitter physiology Koike et al 197 Eisenstadt et al 1973 Kehoe et al 1974 Schwartz 1974 Tauc et al 1974)

Methodological reports on ejectates obtained with various substances labelled radioactive are given in the literature Zieglgänsberger et al (1969) Obata et al (1970) Clarke et al (1973) Hoffer et al (1971) McCaman et al (1977)

In studies on experimentally evoked epilepsy in the neocortex microejection of benzylpenicill

[illegible] in addition to the use of applying penicillin [illegible] ked pledgets on the surface and depth injec[illegible] has been done with iontophoretic technique [illegible] alsh 1971, Curtis et al. 1972).

It has been argued that the iontophoretically [illegible] ected benzylpenicillin influences neuronal mem[illegible] branes or synaptic mechanisms with an excitatory [illegible] result. A further desirable improvement in relating [illegible] penicillin ejectates to functional results, is to quan[illegible] y the ejected volume and relate it to the functional [illegible] ause produced in a neuronal substrate of certain [illegible] ctural organization so as to minimize integrative complexity.

A first step in such an endeavour is to quantify [illegible] penicillin ejectates and to establish, if possible, the [illegible] liability and volume scatter that a chosen pressure [illegible] icroejection technique presents.

As blocking of the pipette is a hazard encoun[illegible] red in any microejection method, benzyl (^{14}C) [illegible] enicillin was used to establish the reliability of the [illegible] ethod and scatter of ejectates. In addition to a few [illegible] ests performed on the relation between pulse dura[illegible] on and ejectate volume, the main interest was [illegible] cused on the volumes obtained on repetition of identical pressure pulses.

By injecting into ten slices in each series, knowl[illegible] dge was obtained on the blocking incidence of the [illegible] pettes [illegible] sed and of the volume scatter in the var[illegible] ous pressure number groups.

The results show that 3 μm pipettes perform, with the pressure parameters used, with an ejection [illegible] ertainty of 99%. If pressure pulses were delivered [illegible] or 4 times, 100% yield was obtained. The aver[illegible] ge ejectate volume obtained from these pipettes, with one pulse was 0.13 nl, corresponding to a [illegible] pherule of about 63 μm diameter. From the [illegible] μm [illegible] ps, ejectate a erages of 0.04 nl (i.e. ~40 μm [illegible] pherules) were obtained. The [illegible] μm pipettes per[illegible] ormed with similar high accuracy, giving an aver[illegible] ge blocking incidence of only 2% at 1 pulse is [illegible] iven. Raising the pulse repetition to 2× 3× or [illegible] × resulted in 100% yield.

The use of [illegible] μm and 5 μm tips subdued to the [illegible] ressure pulses reported here thus enables ejection [illegible] f volumes equal to the sizes of 1-3 neurone soma[illegible] ta in the CA [illegible] region of the guinea-pig hippocam[illegible] pus.

The use of visually checked injection of a sub[illegible] stance into oil gives a good magnitude estimation [illegible] f the amount provided the viewing conditions al[illegible] low high enough accuracy (Chambers & Kopac 1950). If however accuracy in quantification is to be used in functional studies, the visual separation of a sphere of a particular size from that of another is hardly acceptable as distortion of the injected spherule in oil may prevent volume estimation. It is therefore advisable, in future studies, to use the oil injection method as a patency test and for mag[illegible] nitude estimation.

Another point to stress is that penicillin should be injected into an architectonically "simple" nervous structure that allows single neuronal effects to be studied with the least possible circuitry complexity. It may even be possible to study the effect of microejected penicillin on various segments of dif[illegible] ferent membrane areas of single neurones. The cytoarchitectonic feature and the in vitro technique of the transverse hippocampal slice preparation (Skrede & Westgaard 1971) is an excellent struc[illegible] ture for exact studies on the epileptogenic property of benzylpenicillin.

A study of the epileptogenesis of single cells in the guinea-pig hippocampal slice preparation, mi[illegible] croinjected with penicillin, may with the present method thus be conducted with accuracy.

This study was supported by a grant from Medicinska Forskningsrådet, B78-04X-04720-03.

We thank Dr R. Johansson for comments on the manu[illegible] script. A financial support from the Gunvor och Josef Anér Stiftelse is gratefully acknowledged.

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Effect of vagotomy and glucose administration on gastric acid secretion in the Atlantic cod *Gadus morhua*

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HOLSTEIN B & CEDERBERG, C Effect of vagotomy and glucose administration on gastric acid secretion in the Atlantic cod, *Gadus morhua* Acta Physiol Scand 1980 109 37–44 Received 27 July 1979 ISSN 0001-6772. Department of Zoophysiology University of Göteborg, Sweden.

Cods were prepared for measurement of gastric acid secretion. Cannulae were implanted for drainage of the stomach and for separate perfusion of the stomach and intestine Fishes intestinally perfused with diluted sea-water (33% SW) exhibited high rates of acid secretion. This "basal" secretion was abolished (96% inhibition) by bilateral vagotomy Perfusion of the stomach with isosmotic glucose (5.5%) diminished the amount of titratable acid in the gastric effluence, and induced oral ingestion of water Only part of the decrease could be explained by neutralization by ingested sea-water Perfusion of the intestine with 5.5% glucose, containing sea-water to raise the osmolality to that of 50% SW depressed gastric acid secretion compared to 50% SW-perfused controls, without significantly affecting oral intake of water I m. administration of glucose, increasing plasma glucose to the same level as intraluminal administration did not affect acid secretion or oral water ingestion The study demonstrates the existence of potential intestinal mechanism for inhibition of gastric acid secretion, and indicates that vagal activity is independent of the blood glucose level in the cod.

Key words Fish physiology gastric acid secretion, glucose inhibition, vagus nerve

Codfish deprived by pyloric ligation of the possibility to furnish water to the intestine become dehydrated and exhibit high rate of oral water intake and low or no gastric acid secretion. Perfusion of the intestine at a relatively high rate rapidly extinguishes drinking, while perfusion at a slower rate has a less depressant effect on the drinking rate The effects are the same both if the perfusion is dehydrating (pure sea-water 100% SW) or if the perfusion permits the animal to replenish osmotically lost body water (50% or 33% SW i.e. sea-water diluted with one or two volumes of distilled water respectively). When 50% or 33% SW is used for the intestinal perfusion not 100% SW gastric acid secretion is high (Holstein 1979) The mechanism driving this high "basal" secretion is unknown. Since the animals were starved at least one week before experimentation and since hypoglycemia is known to induce vagal activation and ensuing gastric hypersecretion in mammals (Colin-Jones & Himsworth 1969 Stacher et al 1976) we decided to study the effect of vagotomy and glucose administration, intraluminal and intramuscular on codfish basal" acid secretion.

MATERIALS AND METHODS

Animals Codfish of both sexes were used Before experiments they were kept in storage aquaria with recirculating, chilled (10°C) sea-water for at least one week. They were not fed

Surgery The animals were surgically prepared for the separate perfusion of the stomach and intestine, and for drainage of the stomach, by method previously described in detail (Holstein 1979) the cannulae positioned were C1 C2, and C3 In addition some animals were subjected to bilateral vagotomy A small incision was made dorsally in the thin skin behind the posterior gill arch. Through the opening the vagus nerve running in close contact with the duct of Cuvier is accessible The gastric branches were carefully lifted and cut off. Except

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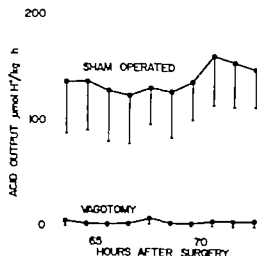


Fig 1 Gastric acid secretion in 5 vagotomized and 5 sham-operated cods Gastric perfusion with 33 % SW At each point the significance for the difference between the groups is $P=0.008$ (Mann Whitney U-test) In this and the following figures means and 1 S E. are given

for cutting the nerves sham-operated animals received the same treatment

Postsurgical treatment Immediately following recovery from anesthesia (MS-222, Sandoz) the fishes were transferred to smaller tanks and perfusion of the intestine (33 % SW) and stomach (100 % or 33 % SW) started The gastric perfusion contained 100 mg/l phenol red (sodium salt Sigma) Perfusion rate for both stomach and intestine was about 8.5 ml/h but varied between pumps and experiments The actual rate was recorded following each experiment Gastric outflow was collected in 1 h fractions. In some vagotomy experiments the intestinal cannula and the pyloric ligation were omitted and perfusion was only via the stomach

The fishes were left without further treatment for at least 20 h and were then used for the experiments during 4 postsurgical days Final body weight was then recorded and expressed as a percentage of the presurgical b wt.

Analyses and presentation of the data

Gastric perfusion The samples were analysed for volume acidity and phenol red (PR) concentration as described earlier (Holstein 1979*a*) From the amount of PR infused and the amount of PR in the gastric effluence the recovery of perfusion fluid was calculated and outflow volume accordingly corrected The acid content was calculated from acidity and corrected volume and expressed as µmol H⁺/kg h in calculating this the presurgical b wt was used In most figures acid secretion has been normalized so that the acid secretion during the hour preceding change of the experimental situation ("control h"), or mean of 5 h preceding change of the experimental situation ("control period") equalized 100 %. Gastric volume outflow is given as a percentage of the hourly infused volume

Blood samples Blood was drawn in heparinized syringes from the caudal vein and the separated plasma samples were frozen until analysed for glucose (glucose oxidase method) osmolality (Wescor 5100R Vapor Pressure Osmometer) K⁺ Na⁺ (Turner Model 510 Flame Photometer) and Cl⁻ (Radiometer CMT 10 Chloride Titrator) concentrations

Statistics The Wilcoxon matched-pairs signed-ranks test the Mann-Whitney U-test the Randomization test for independent samples or the Kruskal-Wallis one-way analysis of variance (Siegel 1956) were used as indicated in text and legends.

RESULTS

Vagotomy Following recovery from anesthesia gastric acid secretion in the sham-operated group gradually increased and at 5–15 h postsurgically the color of the gastric effluence turned to the acid yellow of PR In the vagotomized group the color stayed red for the entire observation period The secretion was assayed in 10 consecutive h as shown in Fig 1 there was an almost complete abolition (i e 96 % inhibition) of acid secretion following vagotomy The mean (±S E) volume of gastric outflow during the 10 h in the vagotomized group (104 71±0 99 %) was not significantly different ($P>0.2$ Randomization test) from the mean volume in the sham-operated group (106 06±0 89 %) Neither were there any significant differences between the two groups concerning b wt or the measured plasma parameters (Table 1)

Experiments were also carried out in animals without pyloric ligation and perfused via the stomach with 33 % SW PR Intact animals exhibited

Table 1 *Some plasma parameters and body-weight (in per cent of presurgical weight) in vagotomized and sham-operated cods*
Means ± S E. of 5 animals each Conc in mM

	[Na⁺]	[K⁺]	[Cl⁻]	mOsm/kg	% b wt
Sham-operated	157 6±6.6	3.8±0 7	141 6±1 9	298 6±6 4	103 6±0 9
Vagotomy	158.2±4.0	3 9±0 7	138.[illegible]±4 4	298 8±6 8	104 4±0 9

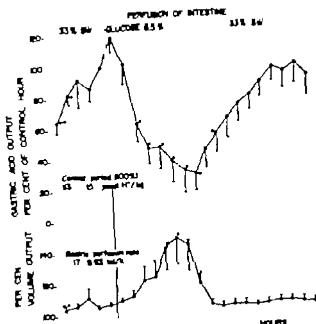


Fig 2 Effect on gastric acid secretion and gastric volume outflow of intestinal perfusion with glucose in 8 animals. In this and the following figures gastric perfusion is with 100 % SW-PR. In the statistical analysis (Wilcoxon test) each point was compared to the control h $P<0.05$ **, $P<0.01$

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Intraluminal glucose perf sion Upon change of the intestinal perfusion from 33 % SW to 5.5 % glucose gastric acid content in the outflow decreased Fig 2) Following rechange to 33 % SW acid out ut recovered and was no longer significantly dif erent from control hour from the 5th posttreatment hour The volume of the gastric outflow increased during glucose perfusion and then, following re change to 33 % SW returned to the basal level. The decrease in acid output was not always associated with increased volume outflow in one fish the volume output (5th h of glucose perfusion) was 106 % et the acid output was depressed to 54 % of the control h, and in another the only slightly increased olume (117 %) occurred with an acid output amounting to 7.5 % of that in the control hour

Effect on plasma glucose and body weight Previous experiments have shown that dehydration inhibits acid secretion and that the % b wt. can be used to measure dehydration (Holstein 1979*a*) To study the effect on b wt. of glucose perfusion these experiments were repeated but the animals now killed following 6 h of intraluminal glucose For comparison, one series in which the 33 % SW perfusion continued for the same time period and another series receiving 5.5 % glucose by the i.m route (0.62 ml/h) were included. Compared to the respective control h intraluminal glucose but not i.m glucose diminished gastric acid output (Fig. 3) The group treated with glucose intraluminally exhibited a small but significant decrease in b wt. (Table 2) Gastric volume output increased (in 4 of the 6 animals) during intraluminal glucose not during i.m glucose (Fig. 3 Table 3). The plasma glucose concentrations were compared to the control group increased in the glucose-loaded groups but the difference between these later groups was not significantly different (Table 4)

Intraluminal perf ion with gluco e/ ea-water The glucose solution used for the intraluminal perfusion contained no salts. Since sodium chloride is

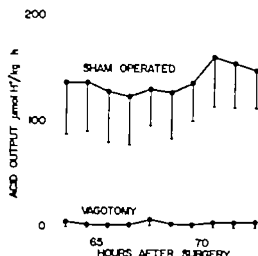


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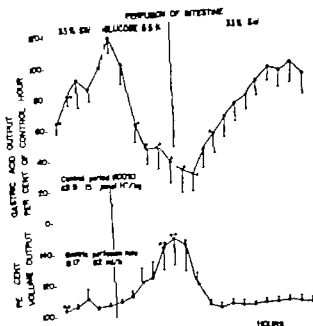


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Intraluminal gluc se perfusion. Upon change of the intestinal perfusion from 33% SW to 5.5% glucose gastric acid content in the outflow decreased (Fig. 2). Following rechange to 33% SW acid output recovered and was no longer significantly different from control hour from the 5th posttreatment hour. The volume of the gastric outflow increased during glucose perfusion and then, following rechange to 33% SW returned to the basal level. The decrease in acid output was not always associated with increased volume outflow: in one fish the volume output (5th h of glucose perfusion) was 106% yet the acid output was depressed to 54% of the control h, and in another the only slightly increased volume (117%) occurred with an acid output amounting to 7.5% of that in the control hour.

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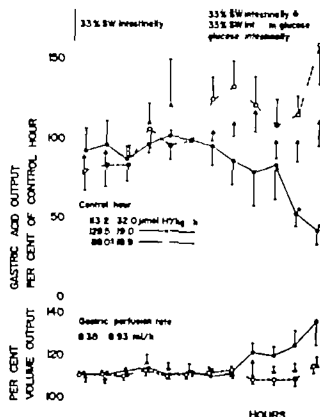


Fig 3 Effect on gastric acid secretion and gastric volume outflow of intramuscular or intraluminal administration of 5.5% glucose P<0 05 for the difference from the control h in the respective series using the Wilcoxon test Further analyses regarding the volume output are presented in Table 3 *n*=6 in each group

essential for the resorption of water from the k the lack of *ions might be responsible for the* ex tered loss of weight The experiment was there modified and the intestine perfused with a mi of 5 5% glucose in distilled water and 5 5% gl in sea water The proportions were such tha osmolality of the mixture equalled that of 50% and then contained 17% SW Fishes perfused *50% SW served as controls Perfusion of the* in tine with glucose-SW inhibited acid secretion (F 4) without significantly affecting volume out (Fig 4 Table 5) volumes surpassing basal c curred only in one of the fishes Again the gluc containing perfusion caused a decrease in b (Table 6) but the final b wt was less than 1 only in 3 of the 6 glucose treated animals.

Correction for neutralization Gastric vol output represents infused volume (*I ml*) and, at basal state s ml corresponding to gastric secretio and water lost to the gastric lumen by osmo (Holstein 1979*b*) The basal output ([I+s/I]×10 was 105–115%

During intestinal perfusion with glucose the v ume output often increases Suppose as is the c during intestinal acidification (Holstein 1979*b*), th this is due to drinking of d ml sea water The v ume output is then (I+s+d/I)×100 expressed in t usual way as a percentage of the infused volum

Table 2. *Body weight in per cent of presurgical weight of glucose-loaded cods (cf Fig 3)*

Group	Treatment	% b wt S E	Statistics
A	Control	102.3±0.5	A vs B NS
B	Glucose i m	104 1±0 7	A vs C P=0 004
C	Glucose i luminally	98 7±0 8	B vs C P=0 00?

Analysis of variance domanstrated a significant (P<0 01) difference among the groups The two-sample compari employed the Mann-Whitney U test

Table 3 *Comparison of gastric mean volume outflow before and during treatment with glucose (cf Fig 3)*

Group	Treatment	Volume outflow (%) Gross mean ± S E	Significance
A	Control 6 h before treatment	111.23±0 80	NS
	Control 6 h during treatment	111 66±1 07	
B	6 h before i m glucose	112 45±1 21	NS
	6 h during i m glucose	114 ±1 46	
C	6 h before intraluminal glucose	112 31±0 7?	P<0 01
	6 h during intraluminal glucose	1 1.53± 77	

Randomization test n=36 in each group (6 animals each)

Table 4 Plasma glucose concentration in glucose loaded cods (cf Fig 3)

Group	Treatment	mg/100 ml ± S.E.	Statistics
A	Control	50.14±3.13	A vs B $P=0.002$
B	5.5% glucose i.m. (34 mg/h 6 h)	264.41±39.18	A vs. C $P=0.002$
C	5.5% glucose i.m. (450 mg/h 6 h)	148.22±52.41	B vs C NS

Analysis of variance demonstrated significant ($P<0.01$) difference among the groups. Two-sample comparisons employed Mann-Whitney U-test

cause to its content of bicarbonate the ingested sea-water neutralizes part of the secreted acid, leading to underestimation of acid secretion. A correction curve was constructed by mixing constant volume samples (i ml) from a pool of gastric effluence with increasing volumes of sea-water (d ml). The acid content was calculated from titrated acidity × total volume (i + d) and plotted against (i + d / i) × 100, i.e. against "per cent volume output". The amount of acid lost by neutralization in the experimental samples was estimated from the correction curve by reading at the percentage volume output following subtraction of (s/i)×100, i.e. the percent increase above infused volume. The obtained value recalculated per kg b.wt was added to the acid output measured as described in the method section. Although this procedure elevated the secretory curves, the effect was slight and only slightly affected the statistical analyses. In Fig. 2, all points marked with asterisks were still significantly different ($P<0.05$) from control but though sometimes at a lower level than before the correction. In Fig. 3, however, the acid output during the final h, though depressed, was no longer significantly different from the control h. In Fig. 4, where the increase in volume was least, the correction did not affect the statistical analyses.

DISCUSSION

Gastric acid secretion in 33% SW-intestinally perfused codfish is high. This work demonstrates that an intact vagal supply is obligatory for this "basal" secretion. Previous results have indicated that a cholinergic mechanism is involved, carbachol stimulates (Holstein 1976, 1977) and atropine and hexamethonium inhibits (Holstein 1979a) codfish acid secretion. Vagal involvement in fish digestive processes has previously been suggested by Russian authors, as cited by Kapoor et al. (1975).

The almost complete blockade of acid secretion following section of the vagi must not be interpreted to indicate that the "basal" secretion is driven solely or even partly by direct vagal innervation. The same results would be expected if vagal activity regulates the release of a stimulating hormone. In mammals gastric acid secretion is regulated by direct vagal routes and by vagus-controlled release of gastrin albeit the relative magnitude varies between species (for references, see Rayford & Thompson 1977). It is also possible that the sen-

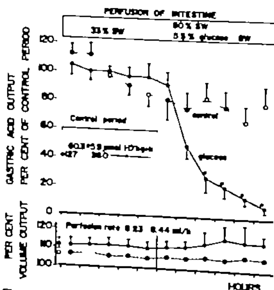


Fig 4 Gastric acid secretion and gastric volume outflow during perfusion of the intestine with 50% S W (n=5) and mixture of glucose and SW isosmotic to 50% SW (n=6). For acid secretion comparisons were between the series. P 0.004 (Mann-Whitney U-test). For volume outflow comparisons were with the respective control periods, indicated to the left (see also Table 5).

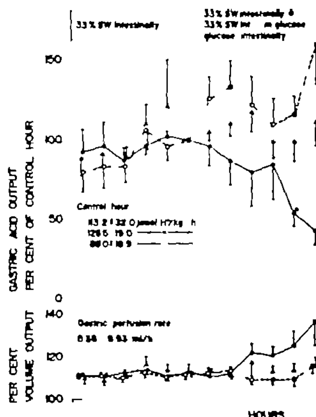


Fig 3 Effect on gastric acid secretion and gastric volume outflow of intramuscular or intraluminal administration of 5 5% glucose $P<0$ 05 for the difference from the control h in the respective series using the Wilcoxon test Further analyses regarding the volume output are presented in Table 3 $n=6$ in each group

essential for the resorption of water from the ... the lack of ions might be responsible for the ... tered loss of weight The experiment was then modified and the intestine perfused with a ... of 5.5 % glucose in distilled water and 5.5 % ... in sea water The proportions were such th... osmolality of the mixture equalled that of 50 ... and then contained 17 % SW Fishes perfused ... 50 % SW served as controls Perfusion of the ... tine with glucose SW inhibited acid secretion ... 4) without significantly affecting volume ... (Fig 4 Table 5) volumes surpassing basal ... curred only in one of the fishes Again the glu... containing perfusion caused a decrease in b ... (Table 6) but the final b wt was less than ... only in 3 of the 6 glucose-treated animals.

Correction for neutrali ation Gastric vol... output represents infused volume (i ml) and ... basal state s ml corresponding to gastric secre... and water lost to the gastric lumen by osm... (Holstein 1979*b*) The basal output ([i+s]/i)×1... was 105–115 %

During intestinal perfusion with glucose the ... ume output often increases Suppose as is the ... during intestinal acidification (Holstein 1979*b*) ... this is due to drinking of d ml sea-water The v... ume output is then (i+s+d/i)×100 expressed in ... usual way as a percentage of the infused volu...

Table 2 *Body weight in per cent of presurgical weight of glucose-loaded cods (cf Fig 3)*

Group	Treatment	% b wt S E	Statistics*
A	Control	102.3±0.5	A vs. B NS
B	Glucose i m	104 1±0 7	A vs C $P=0$ 004
C	Glucose i luminally	98.7±0.8	B vs C $P=0$ 002

Analysis of variance demonstrated a significant ($P<0.01$) difference among the groups The two-sample compar... employed the Mann-Whitney U-test

Table 3 *Comparison of gastric mean volume outflow before and during treatment with glucose (cf Fig ...*

Group	Treatment	Volume outflow (%) Gross mean ± S E	Significance
A	Control 6 h before treatment Control 6 h during treatment	111 23±0.80 111 66±1 07	NS
B	6 h before i m glucose 6 h during i.m glucose	112 45±1.21 114 22±1 46	NS
C	6 h before intraluminal glucose 6 h during intraluminal glucose	11 31±0 72 121 53±2 77	$P<0$ 01

Randomization test $n=36$ in each group (6 animal each)

ance (as indicated by the decrease in b wt.) or to glucose-dependent activation of a nervous or moral reflex mechanism.

Dehydration per se is known to inhibit the ba- secretion (Holstein 1979*a*). Compared to con- ls a decreased mean b wt. was observed in the cose-perfused series but the decrease—though merically significant—was slight in fishes per ed with 5.5 % glucose containing sea-water (Ta- 6, Fig. 4). In this series, marked inhibition was served in all individuals despite the fact that 3 of 6 animals had a final b.wt exceeding the pre rgical weight. Therefore it seems justified to clude dehydration as the main cause of the inhibi- on and we suggest that there exists in the codfish glucose-sensitive, intestinal mechanism for con- l of gastric acid secretion. Previously a similar echanism activated by intestinal acidification has en demonstrated (Holstein 1979*b*).

In mammals, intestinal instillation of hypertonic ucose inhibits gastric acid secretion (Christiansen Hendel 1974, Christiansen et al. 1976, Ward et al. 49). GIP, gastric inhibitory polypeptide, is a po- nt acid secretory antagonist (Pederson & Brown 77) and is released by luminal glucose perfusion arrimer et al. 1978, Sirinek et al. 1977, Sirinek et 1978, Thomas et al. 1977). GIP may prove to be e enterogastrone *responsible for glucose-induced* hibition of mammalian acid secretion (Brown et 1975, Pederson et al. 1978). In fish, however either the existence of GIP nor its effect on acid cretion is known.

The attenuation (low rate perfusion) or extin- uishing (high rate perfusion) of drinking in dehy- rated codfish during intestinal perfusion with pure variously diluted sea-water was interpreted to dicate the presence of intestinal *volume receptors* gulating oral intake of water (Holstein 1979*a*). The ffects are similar to those observed in monkey Maddison et al. 1977), but differs in the respect, at in this species duodenal infusion of saline was effective to stop gastric sham drinking. This may dicate different mechanisms in eliciting preabsorp- ive satiety. For further discussion of this concept is eferred to the *reviews* by Andersson (1978) and itzsimons (1972). A recent report (Holstein 1979*b*) as shown that drinking in the codfish occurs during cidic intestinal perfusion (high rate), and the pres- nt one indicates that this is probably the case also uring glucose perfusion (high rate). The effect may e explained by intense drink-stimulating properties of the infused solutions, or by derepression of the drink reflex. It was thought that drinking might be due to subthreshold concentration in the lumen of some sea-water component, and the inclusion of 17 % SW in the glucose perfusion in diminishing the volume-increase seemed to corroborate this con cept. However the acidic perfusion (Holstein 1979*b*) contained 22 % SW and yet induced a prom- inent but transient volume increase. On the very extreme end perfusion of the intestine for 6 h with distilled water induced a slight (not significant) vol- ume increase without causing any water dificit (un- published observations in 8 animals). Clearly more work is needed before the drinking behaviour dur ing intestinal perfusion can be satisfactorily ex plained. However there still seems to be room for the speculation that ingestion of alcaline sea- water is utilized as an additional means to lower the gastric acidity.

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n = number of h in the gross mean

Treatment	n	Volume outflow (%) Gross mean ± S E	Significance (Randomization test)
5 h before 50% SW perfusion	25	106.60±0.3	NS
6 h during 50% SW perfusion	30	106.70±0.31	
5 h before glucose-SW perfusion	30	111.72±1.26	NS
6 h during glucose-SW perfusion	36	114.38±...1	

sitivity of the secretory cell for other secretogogues is decreased following vagotomy as has been observed in cats and dogs (Andersson & Grossman 1965 Emås & Grossman 1967 Olbe 1963 Svensson & Emås 1977)

Compared to freshly trawled cod (Larsson et al 1976) the plasma glucose level in the present starved control animals appears normal I.m. injection of glucose rendered highly hyperglycemic animals as the glucose level was increased about 4 times but did not affect the secretion of gastric acid. This contrasts to findings in mammals where *artificially increased (by parenteral administration)* blood glucose levels inhibit both basal and postprandial secretion (Dotevall & Muren 1961 Kemp et al 1968 Moore 1973 Solomon & Spiro 1959 Stacher et al 1976) though concerning pentagastric-induced secretion the results are conflicting (Holst & Christiansen 1975 Markiewicz & Lukin 1976) The mammalian lateral hypothalamus contains glucose-sensitive chemoreceptors (Colm-Jones & Himsworth 1970) the inhibition probably beeing mediated via these structures Our results seem irreconcilable with the existence of a similar mechanism in the cod Also in the chicken the response of acid secretion to changes in metabolizable glucose levels deviates from that in the mammal (Burhol & Hirschowitz 1971 Ruoff & Sewing 197) In another fish the blood glucose level has been shown to regulate appetite (Kuzmina 1967) the present results do not exclude such a mechanism in the cod

Perfusion of the intestine with isosmotic glucose elevated the plasma glucose to a level comparable to that following i m administration but in contrast to the i m route intraluminal glucose depressed the acid output The interpretation of this result i complicated by i) the increased volume output and ii) the decreased b wt accompanying glucose perfusion The increased volume may reflect a greater volume flow (active secretion or passive flux along the tissue-to-lumen osmotic gradient) from the mucosa or ingestion of water A similar increase in gastric volume outflow was observed during acidic perfusion of the intestine this increase was due to oral ingestion of water (Holstein 1979b) By analogy we consider the "drinking alternative the most likely If this is correct the neutralization of secreted acid by sea-water has to be considered A correction was applied and found to have only minor influence on the results Moreover during *intestinal glucose perfusion* the decrease in acid output was not always accompanied by increased volume output When sea-water was included in the glucose perfusion (total osmolality equal to 50% SW) the volume increase was insignificant but acid output still declined Hence it is concluded that the major part of the observed decrease in acid output represents a glucose-induced inhibition and the neutralization may contribute slightly It is also concluded that the inhibition is not related to the osmolality of the luminal content though the inefficiency of i m glucose shows that the inhibitor emerges from the intestine However the inhibition may be due either to interference with the wate

Table 6. *Body weight in per cent of pre-experimental weight following 6 h of intestinal perfusion with 50% SW or a sea-water containing 5.5% glucose solution is ... m ... t 50% SW (cf Fig 4)*

Group	% b wt ± S E	P (Mann Whitney U-test)
50% SW perfusion	10_.8±0.9	0.018
5.5% glucose SW perfusion	100.0±0.6	

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asomotor effects of facial nerve stimulation oncholinergic vasodilation in the eye

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STJERNSCHANTZ, J & BILL, A. Vasomotor effects of facial nerve stimulation, noncholinergic vasodilation in the eye. Acta Physiol Scand 1980, 109 45–50 Received 9 Aug. 1979 ISSN 0001-6772. Institute of Physiology and Medical Biophysics, University of Uppsala, Sweden.

The effect of facial nerve stimulation on ocular blood flow was studied in rabbits. The intracranial part of the facial nerve was stimulated electrically and the regional blood flow was measured with labelled microspheres. Effects on the intraocular pressure were determined in a separate series of experiments. Stimulation increased choroidal blood flow by about 200%. The blood flow of the iris and the ciliary processes also increased. The blood flow of the eyelids and the nictitating membrane increased by approximately 1000% and the blood flow of Harder's gland increased by about 200%. The blood flow of the tongue and the submandibular gland also increased. The increase in the uveal blood flow could not be abolished by a cholinergic or ganglionic blockade. Ganglionic blockade abolished most of the increase in the blood flow of the eyelids, nictitating membrane and Harder's gland, the cholinergic blockade seemed less effective. The intraocular pressure increased with a mean value of 6 mmHg during optimal (20–30 Hz) stimulation. The increase could not be prevented by cholinergic blockade. Much of the increase in uveal blood flow seemed to be caused by stimulation of unconventional nerves. It is suggested that these nerves may be peptidergic using VIP as transmitter and lacking a hexamethonium sensitive synapse between the site of stimulation and the eye. Their nature—afferent or efferent—remains unknown. A great part of the increase in blood flow of the eyelids, nictitating membrane and Harder's gland seemed to be due to other mechanisms involving nerves with a peripheral synapse.

Key words. Facial nerve, blood flow, intraocular pressure, uvea, eyelids, nictitating membrane, Harder's gland, submandibular gland, tongue

Very little is known about the influence of the facial nerve on ocular blood flow and aqueous humor dynamics. According to Ruskell (1965, 1970a) non-adrenergic nerves from the sphenopalatine ganglion of facial nerve origin distribute to the eye in the rabbit, monkey and man. At least in the monkey a considerable part of these nerves seems to terminate in the wall of arterioles in the choroid (Ruskell 1971), which indicates that they may influence ocular blood flow. Intraocular pressure (IOP) has been reported to increase upon stimulation of the greater superficial petrosal nerve in cats and rabbits (Gloster 1961) and interruption of the facial nerve pathway seems to cause a reduction in IOP (Ruskell 1970b).

The facial nerve has been regarded as containing vasomotor nerves for the lacrimal and nasal glands, the sublingual and submandibular glands and the tongue. Erici & Uvnäs (1951) showed that stimulation of the chorda-tympani nerve in cats induces vasodilation in the tongue probably due to efferent fibres. Hellekant (1977) by stimulating the chorda-tympani proper nerve in rats was able to demonstrate a significant decrease in the constant flow perfusion pressure of the tongue, which was due to stimulation of efferent nerve fibres according to classical criteria. Eccles & Wallis (1974) also demonstrated vasodilation in the tongue with a plethysmographic technique during stimulation of the lingual nerve in cats.

Stimulation of the chorda-tympani nerve in cat was demonstrated as early as 1858 by Bernard to

induce vasodilation in the submandibular gland This effect was analyzed recently by Gautvik (1970a b c) The vasodilation of the tongue and the submandibular gland induced by nerve stimulation was very atropine resistent (Enel & Uvnäs 1952 Gautvik 1970c)

The purpose of the present investigation was to study the effect of stimulation of the facial nerve on the blood flow in the eye and the intraocular pressure Some other organs have also been studied primarily in order to check the selectivity of the stimulation The possibility that co-stimulation of the trigeminal nerve was involved in the effects observed was also investigated

MATERIALS AND METHODS

Albino rabbits of either sex weighing 1 7–3 0 kg were used The animals were anesthetized with i v pentobarbital sodium 40–50 mg/kg b w and were artificially ventilated The P_{CO_2} P and pH of the arterial blood were checked and the ventilation adjusted so that normal acid–base levels resulted Both femoral arteries were cannulated one for blood sampling and the other for blood pressure measurement The animals were thoracotomized and the left heart ventricle was cannulated All cannulas were heparinized Whenever needed a mixture of Macrodex (Pharmacia Uppsala) and isotonic saline (1 1) was infused intravenously to increase the blood pressure The amount of this mixture did not exceed 40 ml The animals were kept warm with a heating pad

Intracranial stimulation of the facial nerve

The facial nerve was stimulated intracranially at the internal acoustic pore with stereotaxic technique The skull of the animal was firmly fixed in a stereotaxic frame A small hole was bored in the skull at the sagittal suture so that the electrode could be inserted obliquely through the sagittal suture or 0.5 mm laterally at the side to be stimulated The electrode was inserted 6.5–8.5 mm posterior to the bregma at a 16 lateral angle to the vertical axis 21–24 mm obliquely inferior—calculated from the upper surface of the skull bone at the bored hole The coordinates were calculated for the rabbit head which was in a slightly backward leaning position (10–15°) in the stereotaxic instrument The electrode was left in the position where good lacrimation could be obtained In most of the experiments lacrimation was combined with salivation Stimulation was carried out with unidirectional square wave pulses through a bipolar electrode The distance between the poles of the electrode was 1 mm Stimulation parameters were pulse intensity 6–14 V duration 1 ms and frequency ranging from 5 to 60 Hz, mostly being 40–50 Hz. The nerve was stimulated for 4–5 min After the experiment the position of the electrode was checked Four experiments were performed with facial nerve stimulation after intracranial transection of the trigeminal nerve made 5–8 mm anterior to the place where the nerve enters the middle cerebral fossa. The skull was widely opened and p[illegible] of the hemisphere had to be removed before transectio[illegible]

Table 1 *Effect of facial nerve stimulation on [illegible] blood flow*

Indicates $P \leq 0.01$ and $P \leq 0.001$ $n = 18$ Differe[illegible] were calculated from paired data

Tissue	Stimulated side (mg/min)	Control side (mg/min)	Difference (mg/min)
Choroid	2 413±237	900±106	1 513+[illegible]
Iris	157±19	90±15	67±[illegible]
Ciliary processes	106±10	81±11	25+[illegible]

Determination of regional blood flow

Regional blood flow was determined with 15 µm radioactively labelled microspheres (3 M Company) according to the reference flow method (see Alm & Bill 1972) The microspheres were injected into the left heart ventricle Each animal received 2–3 separate injections with different labelled microspheres It was thus possible to determine the blood flow at two to three different times during the experiment e g. during stimulation before pharmacologic blockade after pharmacologic blockade and after pharmacologic blockade during stimulation. The method is described in more detail elsewhere (Stjernschantz, [illegible] & Bill 1976) The tissues studied were the choroid [illegible] ciliary processes scraped off the back side of the iris [illegible] the iris the eyelids the nictitating membrane Harderian gland the submandibular and parotid glands the tongue the masseter muscle and the facial skin The tissue [illegible] blood samples were assayed in a three-channel gamma spectrometer The blood flow in the choroid ciliary processes and the iris is presented as the blood flow of the whole organ (mg/min) while that of the other organs [illegible] presented as the blood flow per g tissue (mg/min/g)

Pressure measurements

The intraocular and blood pressures were measured with pressure transducers The eyes were cannulated using needle gun and a slow infusion (0 6 µl/min) of an electrolyte solution (Bárány 1964) was used to keep the cannula open This only slightly affected the IOP After each stimulation of 3–5 min the IOP was given time to return to the baseline and stabilize for 5–10 min before the next stimulation The animals used for IOP measurements did not receive any sphere injections and consequently were not thoracotomized

Pharmacologic blockade

All the animals underwent neuromuscular blockade with tubocurarine 0.5–1 mg/kg b wt i [illegible] administered in order to avoid movement of the eyelids during stimulation Cholinergic (muscarinic) blockade was induced by biperiden 2.5–10 mg/kg b wt i [illegible] administered Ganglion blockade was carried out with hexamethonium 50–1[illegible] mg/kg b wt i v administered

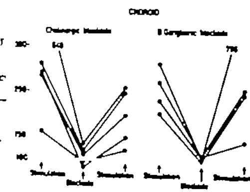


Fig 1

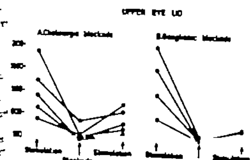


Fig 2

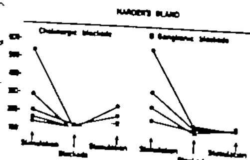


Fig 3

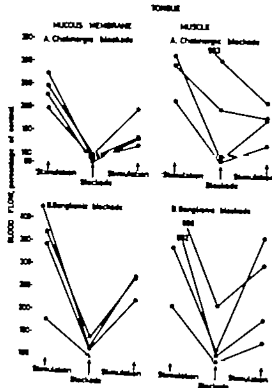


Fig 4

Figs. 1-4 Effect of pharmacologic blockade on the vasomotor effect of facial nerve stimulation. The blood flow on the stimulated side was expressed as percentage of that on the control

ESULTS

timulation caused lacrimation and in most of the xperiments salivation. A white lipidic substance a also secreted probably from the eyelids. Lac mation and salivation were totally prevented by holinergic and ganglionic blockade but transec on of the fifth cranial nerve prior to stimulation of e facial nerve did not affect the responses Stimu ation had no appreciable effect on the pupil size nd caused little change in the blood pressure

The effect of facial nerve stimulation on the uveal ood flow is presented in Table 1 The mean in rease in the choroidal blood flow was 1 ±38% ($P<0\ 001$ paired data) The increase in blood flow in the iris and ciliary processes was less but statistically significant at the $P<0\ 01$ level Fig. 1 shows that cholinergic blockade with biperiden did not adequately abolish the increase in the choroidal blood flow during stimulation Ganglionic blockade with hexamethonium was in a similar way ineffective (Fig. 1B) Transection of the fifth cranial nerve 60–90 min before stimulation of the facial nerve did not prevent the vasomotor response The blood flow in the iris and the ciliary responded in a similar way to that of the choroid after cholinergic and ganglionic blockade and after transection of the fifth cranial nerve

Table 2 *Effect of facial nerve stimulation on the blood flow in tissues innervated by the facial nerve* * indicates $P<0.05$, $P<0.005$ and $P<0.001$ The mean difference was calculated using paired data

Tissue	n	Stimulated side (mg/min/g)	Control side (mg/min/g)	Difference (mg/min/g)
Upper eye lid	18	1 295±199	158±33	1 136±182
Lower eye lid	18	1 174±70_	153±33	1 021±191
Nictitating membrane	18	986±164	100±17	887±149*
Harder's gland	18	1 012±193	380±91	63 ±176
Submandibular gland	10	756±1 2	322±70	434±146
Tongue (muc membr)	17	1 578±353	48_±6_	1 097±316
Tongue (muscle layer)	17	217±61	53±7	164±62

The blood flow of the upper and lower eyelids and of the nictitating membrane increased almost ten times (Table 2) The blood flow of the mucous membrane and the muscle layer of the tongue also strongly increased and the same was true for the blood flow of the submandibular gland in the 10 experiments in which there was a clear salivation (Table 2) Contrary to this the blood flow of the parotid gland masseter muscle and the facial skin showed only minute changes during stimulation

Cholinergic blockade partly inhibited the increase in the blood flow of the upper eyelid and of Harder s gland (Fig 2 A and 3 A) but ganglionic blockade with hexamethonium inhibited most of the increase in the blood flow of these structures during stimulation (Fig 2 B and 3 B) Transection of the fifth cranial nerve 60–90 min before stimulation of the facial nerve did not change the response to stimulation The blood flow of the lower eyelid and the nictitating membrane responded in a similar way to that of the upper eyelid and Harder s gland after cholinergic and ganglionic blockade and after transection of the fifth cranial nerve In the tongue stimulation of the facial nerve caused dilation even after cholinergic and ganglionic blockade (Fig. 4) The arterial blood pressure during stimulation after cholinergic blockade was 74±6 mmHg and the corresponding value after ganglionic blockade was 70±6 mmHg

Stimulation of the facial nerve caused a rise in IOP of 5 9±1 3 mmHg at 20–30 Hz higher frequencies did not further increase the pressure (Fig. The IOP before the injection was 15–20 mmHg The increase in IOP was essentially unilateral there was a slight change in IOP on the contralateral side only when stimulation affected the blood pressure It rapidly increased upon stimulation The maximal value reached within 10–20 s was usually followed by a slight fall in IOP After stimulation the return of IOP to the prestimulation level generally took 30–60 s A typical result is shown in Fig Cholinergic blockade with biperiden did not prevent the increase in IOP

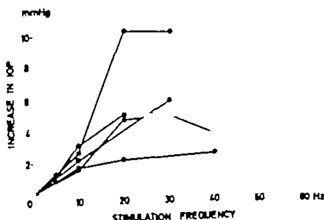


Fig 5 The relationship between stimulation frequency and a change in the IOP during facial nerve stimulation.

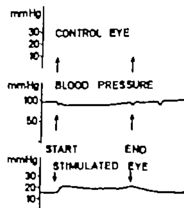


Fig 6 IOP and blood pressure measurement in a typical experiment Note the abrupt and somewhat transient increase in IOP upon stimulation Stimulation period

[DIS]CUSSION

[Stim]ulation caused lacrimation and in most experi[ments] salivation which indicates that the facial [ner]ve was stimulated The selectivity of the stimu[lati]on is demonstrated by the fact that tissues [kn]own to be innervated by the facial nerve re[sp]onded with clear change in blood flow while control tissues showed no or only minute [ch]anges during stimulation. Thus, co-stimulation of [oth]er structures interfering with the results seems [im]probable Nevertheless, if there was a co-stimu[lati]on the most suspected structure with regard to [the] observed results would be the trigeminal nerve [El]ectrical stimulation of the trigeminal nerve has [be]en shown to cause release of substance P in the [ey]e (Bill et al. 1979) and an increased uveal blood [flo]w and increased blood flow of the upper eyelid [an]d buccal skin (Stjernschantz, Geijer & Bill 1979) [Si]nce transection of the trigeminal nerve was with[ou]t effect in preventing the vasomotor response to [fac]ial nerve stimulation, it may be assumed that no [su]ch co-stimulation took place.

Stimulation caused a marked increase in the uve[al] blood flow especially in the choroidal blood [flo]w Since cholinergic as well as ganglionic block[ad]e did not prevent the increase in uveal blood [flo]w the stimulated nerves can hardly be cholinergic [fib]res with a synapse in the sphenopalatine gang[lio]n, they rather seem to be of an unconventional [ty]pe Substance P is unlikely to be the transmitter [si]nce this agent causes miosis (Bill et al 1979) [w]hich was not observed in the present experiments. [In]terestingly nerves containing VIP (Vasoactive [In]testinal Peptide) have recently been found in sev[er]al orbital structures in cats. The nerves were in [clo]se contact with the small vessels of the choroid [th]e ciliary body the lacrimal and tarsal and Har[der]ian glands and VIP immunoactive cell bodies [we]re found in the pterygopalatine ganglion [(U]ddman et al. personal communication) VIP [ca]uses vasodilation in the choroid and at high doses [so]me salivation (Bill & Nilsson, unpublished re[sul]ts). It seems quite possible then that the uncon[ven]tional nerves involved in the vasomotor re[sp]onses reported here are peptidergic using VIP as [a] transmitter

The mechanism resulting in an increased blood [flo]w in the eyelids, the nictitating membrane and [H]arder's gland seemed to be different from that in [th]e choroid Most of the vasodilation in these tis[su]es could be abolished by ganglionic blockade and by cholinergic blockade The eyelids of the rabbits contain secretory elements. It is of interest then that in Gautvik's studies (1970*a b c*) the vasodilation obtained in the submandibular gland on chorda-lingual nerve stimulation was due partly to a direct nervous mechanism on the blood vessels and partly to activation of the glandular structures causing release of kinins. Moreover in recent studies by Uddman et al (personal communication) release of VIP was observed from the submandibular gland on lingual nerve stimulation. It thus seems that facial nerve stimulation may have direct effect on glands and blood vessels mediated by acetylcholine and VIP and indirect effects mediated by kinins.

Lingual blood flow also markedly increased during stimulation which is in good agreement with the results of Erici & Uvnäs (1957) Eccles & Wallis (1974) and Hellekant (1977) In earlier studies it was not possible to distinguish between the blood flow of the muscle layer and that of the mucous membrane. Thus it was interesting to note in the present experiments that the blood flow in each of the structures increased during stimulation These nerve fibres may also be peptidergic since ganglionic blockade was ineffective in abolishing the response

It can be seen from Fig. 1–4 that in some experiments the second flow determination made after a period of 70–115 min with no stimulation indicated flow values on the electrode side that were higher than those on the control side This result suggests that the period of time without stimulation was not long enough to give a complete return to prestimulation levels. One can speculate that the reason for the poor return in the tongue muscle may be that in tissues with continuous capillaries peptide transmitters with a relatively high molecular weight will tend to have a longlasting effect due to poor elimination via the blood stream.

In the present experiments the intraocular pressure increased moderately during facial nerve stimulation. Gloster (1961) when stimulating the greater superficial petrosal nerve in cats and rabbits also registered a moderate increase in IOP This effect was resistent to atropine and was thought to be due to vasodilation in the eye. This hypothesis is supported by the present results The initial rapid increase in IOP in the present study probably was due to vasodilation resulting in an increased intraocular blood volume as well as an increased blood flow Whether facial nerve stimula-

tion has other effects in the eye remains to be studied

The observation in the present experiments that repeated 3–5 min stimulations were effective is of interest since it indicates that there is no rapid depletion of the transmitter from the nerve terminals This of course is a prerequisite for the proper function of the peripheral terminals of efferent nerve fibres Further studies are required however to ascertain whether the nonmuscarinic responses observed in the present study are mediated via afferent or efferent nerve fibres

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The ultrastructure of the gastric glands and its relation to induced secretory activity of cod *Gadus morhua* (Day)

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MATTISSON A & HOLSTEIN B The ultrastructure of the gastric glands and its relation to induced secretory activity of cod, *Gadus morhua* (Day). Acta Physiol Scand 1980, 109 51 59 Received 13 Aug. 1979 ISSN 0001-6772. Department of Zoophysiology University of Göteborg, S eden.

Light and electron microscopic studies have been performed on different parts of cod stomach. The studies have been concentrated on the gastric glands of the gastric mucosa. The glands consist of only one cell type This cell contains an apically located comprehensive tubular system as ell as zymogen granules and therefore probably secretes both acid and zymogen The high degree of development of certain organelles and their mutual localization shows structural organization which appears suitable for an effective secretion. What appears to be intermediate organelle forms are common. When acid secretion is increased by continuous intramuscular infusion of histamine or of carbachol the most marked structural changes are increases in the size of the gland lumen and in the cell membrane facing the lumen. Histamine in particular causes the appearance of numerous long and slender cell processes in an enlarged gland lumen When the acid secretion increased by histamine, had reached plateau metiamide was infused This histamine H_2-receptor antagonist caused decrease in acid secretion as well as in the size of the gland cell surface to values characteristic of untreated cod

Key ords Gastric glands ultrastructure acid secretion, cod

From studies on different organisms it is well known that histamine strongly stimulates gastric acid secretion (Popielski 1920, Keeton et al 1920, Friedman 1937 Grzyym et al. 1968) and that metiamide reduces or eliminates this effect of histamine (Black et al 1973 Gibson et al 1974 Konturek et al 1974) Recently Holstein (1976) has examined the effect of histamine, metiamide and carbachol on the gastric acid secretion from the Atlantic cod *Gadus morhua* Histamine and carbachol were found to increase the acid output the effect of both agonists was blocked by metiamide.

The present investigation complements the above mentioned studies on the acid secretion of cod stomach. It comprises light- and electron microscopic studies on different parts of cod stomach The purposes are ( ) to give the structure and ultrastructure of the cod stomach and (*b*) to show structural and ultrastructural changes which can be related to a changed acid secretion caused by histamine by a subsequent addition of histamine together with metiamide and by carbachol alone

The histology of the stomach of cod has been described by several authors (Thesen 1890, Greene 1912, Blake 1930 Rogick 1931 Bishop & Odense 1966) Gastric function in fish has been treated in recent reviews (Kapoor et al 1975 Fänge & Grove 1979) We have found no previous electron microscopic studies on the stomach of cod.

MATERIAL AND METHODS

Cod were kept in aquaria ith running sea water for 1–2 weeks before the xperiments were performed The cod were all around 30 cm long N regard was taken to different sexes as their secretory activities are similar Untreated fish were udied to make comparisons. The experimental fish prepared for collection of gastric secretions as described earlier (Holstein 1975) were treated with continuous intramuscular infusion either of carbachol 5 µg/kg h, or of histamine 15 µg/kg h For some fish treated with histamine after the secretory rate had attained plateau, metiamide 2.44 mg (10 µmol)/kg h was superimposed on the histamine injection. Within 1 3 min after stunning the fish samples for fixation were taken from three regions of the internal stomach wall, i.e. the uppermost part and about one third and two thirds down

the stoma h length Our main interest concerned the re gion one third down the stomach length from the oesophagus

Histology Pieces of isolated mucosa were fixed and embedded either by routine methods for light microscopy or as described below for electron microscopy Staining was done with toluidine blue or with eosin haematoxyline A modified Zimmerman technique (Marks & Drysdale 1957) was also used

Electron microscopy The best fixation was obtained with 3% glutaraldehyde in 0 1 M sodium-cacodylate buffer (pH 7.3) made up to a final concentration of 5% with sucrose and adding a trace of CaCl (cf Bell et al 1969) Post-fixation was done in 1% OsO in 0 1 M veronal acetate buffer at a pH of 7 4 For some specimens this OsO solution was the only fixative used Dehydration in increasing concentrations of ethanol was followed by embedding in Epon 812. Sections were cut with an LKB ultrotome set at 100 to 500Å Contrast was obtained by using uranyl acetate followed by lead citrate An Hitachi HS-8 electron microscope was used and gave magnifications from ×1900 to ×47 000

RESULTS

Histology As has been reported by some authors e g Blake (1930) the gastric cells of teleosts often do not take or take only lightly the ordinary cytoplasmic stains In our study the best results were obtained with toluidine blue when used for ½–1 μm thick sections cut from tissue embedded in Epon 81? Thinner sections used for low power electron microscopy gave complementary information about the microscopic characteristics of the stomach wall

The layers of the stomach wall are in order mu cosa submucosa muscle layer and serosa The gastric mucosa consists of surface epithelium and a connective tissue layer containing the gastric glands

The surface epithelium is composed of columnar cells which reach their maximum length in the upper and middle part of the stomach Among the columnar cells are goblet cells which due to their translucent vesicles appear light in the electron mic roscope and are less densely stained by toluidine blue Both of these cell types have microvilli but those of the goblet cells are short and more scat tered The cylindrical epithelium cells when they descend the pits show a gradual transformation to cuboidal neck cells These latter ones show special staining properties (cf Leeson 1975*a*)

Below the epithelium there is a thick connective tissue layer Within this layer and well separated from the epithelium there is a region containing the gastric glands The glands converge in groups to-



Fig 1 Section through stomach wall showing the gr mucosa Lumen (*l*) gastric pit (*p*) and the glan which occur in a thick connective layer The ga glands emerge with common openings into the gastric Epon section stained with toluidine blue

wards a common passage which emerges into a of the gastric wall (Fig 1) Among the glands t connective layer is intervowen with blood ca laries and some muscle cells. Within the submuc no distinct muscle layer is developed

Electron microscopy As in most other f studied (Blake 1930 Rauther 1940 Hale 1965 Li & Tan 1975 Noaillac Depeyre & Gas 1978) and most other non-mammalian vertebrates (Sel 1962 Leeson 1975a Wight 1975) the gastric gl of the cod contain only one type of cells suppos to secrete both pepsinogen and hydrochloric ac This type of cell usually is referred to as ox topeptic (Barrington 1942 Smit 1968) In tra verse section 5–8 such cells build up each gastr gland (Fig .) The cells are pyramidal with t narrow tops bordering the lumen of the gland Th boundaries between the cells are enlarged and u dulated and form spaces between the cells whi probably are of importance for the secretory fun tion of the cells (Ito 1961 Kaye et al 1965)

Within the cells 4 components predominat vesicles more or less filled with a rather den homogeneous material well developed mitoch



Fig. 2 Electron micrograph of a transverse section through gastric gland from untreated cod The cells are separated by undulating canals. In the gland lumen (*l*) there are microvillous processes and an electron dense material appearing like the contents of the zymogen granules. The cells are filled up with mitochondria, zymogen granules, and apically arranged smooth tubules. The rough endoplasmic reticulum makes the cell bases more dark I the connectiv layer blood vessels (*b*). Fixation glutaraldehyde osmium

ia, smooth densely packed tubules and a rough ndoplasmic reticulum The nuclei are located in e basal part of the cells The vesicles are of about e same size—around µm n diameter—and are niformly scattered over the cell (Fig. 2) Close to e lumen of the gland the esicles are often seen to lease their contents by a process of exocytosis Fig. 6) It is common to find former vesicular cont in the gland lumen as aggregates mostly enlosed by thin and broken membranes (Fig. 3) Occasionally zymogen granules as such seem to be xtruded into the gland lumen (In Fig 3)

The mitochondria are scattered all over the cell but are more numerous towards the periphery of the glands Mostly the mitochondria are densely packed with cristae and generally contain electron dense granules (Figs. 4a 6) In some cases, how ever only portions of the mitochondria contain cristae while the rest consists of a relatively dense zymogenlike matrix or is entirely translucent (Fig. 4b 6) Some of these structures often give the impression of being intermediate forms between mitochondria and zymogen granules (cf Sedar 1961). The high frequency of these structures indicates the existence of transition forms. Cod injected with histamine alone, often show mitochondria with



Fig 3 Electron micrograph of apical portion of a gland cell from untreated cod In the lumen a zymogen granulum ( ) together with zymogen partly enclosed by a thin membrane This membrane also fragmentarily borders the lumen At arrows there are indications of openings of the smooth tubules into the lumen Glutaraldehyde-osmium

a reticular conformation of the cristae Marked mitochondrial changes also occur in glands from animals treated with carbachol most of the mitochondria show a compartmentalisation and the different regions have their special arrangement of cristae

Irrespective of the treatment of the cod the smooth tubules predominate in the apical part of the gland cell (Figs 2 and 5) The smooth tubules often show a very intimate contact with the mitochondria as well as with the zymogen granules Several surface views give the impression of tubules passing the membranes of these organelles (Figs 4*a* 5 6) Apically the tubules often show an apparent communication with the gland lumen (Figs 3 5) This gives the possibility that acid is added to the gastric juice by an evagination process

The rough endoplasmic reticulum is well supplied with ribosomes and generally occupies a great portion of the cell basis Mostly it is in close association with the mitochondria and the zymogen granules and fills up the space between these ganelles (Fig 4*b*) Due to the contrast agents the rough endoplasmic reticulum gives the basis a high degree of electron density

In untreated animals the gland cell surface wards the lumen shows some short processes the surfaces bordering neighbouring cells are lated (Fig 2) The most marked structural ch of the gland cells when treated with histamine is enormous increase of the apical cell surface (F 5) Moreover the gland lumen is increased. A gr number of long and thin processes occupy most the lumen and in some pictures at least also t intercellular boundaries show an increased ir lated arrangement A coincidental reduction of t amount of the smooth tubular system as repor for several organisms when treated with histam (Vial & Orego 1960 Sedar 1961 1962) is not ob ous in glands of histamine-treated cod

The previous observation that metiamide s presses histamine-induced gastric acid secre (Holstein 1976) was confirmed in the present inv tigation Comparing histamine-treated cod w those injected with both histamine and metiam the latter have a great reduction in surface a towards gland lumen This surface is of about t same size as that of untreated cod (Fig 6)

The secreted zymogen occurs as a semi-de mass in the lumen of the gland Estimation of t sectioned area of the zymogen in relation to t luminal surface in a number of glands (38) gave i indication of the variation in amount of secre zymogen Histamine stimulates acid secretion a increases the size of the gland lumen In spite that the zymogen areas did not decrease This i cates an increase of zymogen secretion also. Us a modified technique in which the fish wa supplied with water in the intestine (Holstein 197 Cederberg (unpublished) by studying the proteas activity of the gastric outflow found that histam increased the pepsin activity A similar increase h been reported for the western newt (Leeson 1975) In fish exposed to a combined treatment with hi tamine and metiamide the increased zymogen s cretion obtained with histamine alone often see to persist for a period of some hours (Fig. 6)

Cod treated with carbachol also show an incre in gland cell surface though less marked than i those injected with histamine As reported by Ho stein (1976) the increase in acid secretion was al less with the concentrations of carbachol used

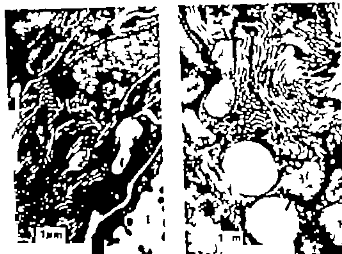


Fig 4 Portions of gland cells from cod continuously supplied with histamine ( ) At some places (large arrow) one might get the opinion that tubules penetrate into mitochondrion. Often these tw organelles show an intimate contact. I the lumen (*l*) the typical thin membranes. (*b*) Possible intermediate forms between mitochondria and zymogen granules at small arrows Glutaraldehyde-osmium.



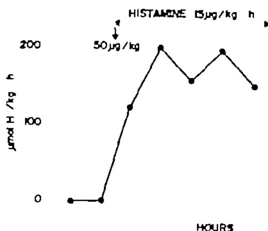
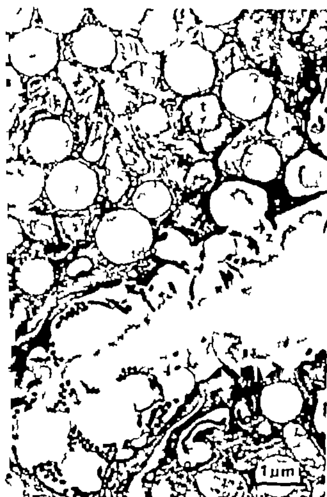


Fig 5 Apical portions of gland cells of cod treated with histamine An increase of gland lumen and an enormous increase of cell surface towards lumen are obvious. Apparent close associations between mitochondria and smooth tubules at small arrows Indications of openings of the tubules into the lumen at large arrows. Graph shows acid secretion against time Where the curve ends the cod was killed. Glutaraldehyde-osmium.



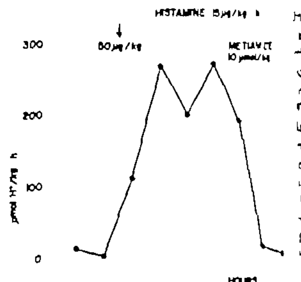


Fig 6 The lumen region of a gland of cod supplied histamine superposed by metiamide Metiamide cause reduction of the number and size of the processes at lumen The lumen is filled up with zymogen and membranes At a zymogen granule empties. Few transition form between zymogen granules and mitochondria at *l* Dense granules occur within several mitochondria and at *t* a translucent region At arrow smooth tubules seem to enter a granule supplied mitochondria Graph gives the variations of acid secretion during the experiment When the curve ends the cod was killed Glutaraldehyde-osmium

DISCUSSION

The stomach wall of cod is built up of layers in a similar way to that described for mammalian stomach In cod however there is no distinct muscle layer within the submucosa Muscle cells occur scattered in the connective layer as has been reported for some other fish (Noaillac Depeyre & Gas 1978)

The structure and ultrastructure of the gastric glands of cod show similarities with those of other lower vertebrates As reported for reptiles (Ferri et al 1974) for amphibians (Sedar 1962) and for some fish (Western & Jennings 1970 Ling & Tan 1975 Noaillac Depeyre & Gas 1978) the cod gastric glands contain one type of cells only These cells have secretory granules or vesicles as well as a vast tubular system and we suggest that they have the function of secreting both digestive proenzymes and the acid The vesicles probably contain and release a proenzym with proteolytic activity A pepsinic activity in gastric juice has been shown in some fish (Norris & Mathies 1953 Norris et al 1973 Ozgyan et al 1968 Smit 1967) The same tubular system predominating in the apical portion of the gland cells resembles that of amphibian "oxyntopeptic" cells and though lacking canaliculi that of mammalian parietal cells This system seems to open towards the gland lumen and then in reality to form an immense cell surface Several authors working with different animals (Kasbekar et al 1968 Lillibridge 1968 Sedar 1969 Leeson 1972 1974) have suggested that the tubules form part of the cell membrane A vast surface of a similar kind is common for cells with a high ion exchange e g the chloride cells and some pseudobranch cells of fish (Copeland & Dalton 1959 Philpott & Copeland 1963) and it is an attractive hypothesis that the tubules function as a store for acid. We expect that when acid secretion is stimulated by drugs the tubular system would bulge out into the lumen and give rise to the numerous processes so characteristic of the stimulated cells Further studies are planned to test this hypothesis

The cell surface facing the lumen is covered with

...un membrane which generally joins the proces- and also the zymogen when it is extruded The ...mbrane appears like the peritrophic membrane ...cribed from the intestine of an insect *Triatoma ...fans* by Burgos & Gutierrez (1976) Leeson ...15) described the occurrence of a membrane glycocalyx or "fuzz" on the microvilli and the ...al plasma membrane of the stomach of the ...tern newt He also suggested a release of intact ...nogen vesicles into the lumen of the gastric gland ...owing histamine stimulation. In the cod the ...mbrane enclosing zymogen is generally thin and ...he same type as that covering the cell surface ...ugh occasionally whole zymogen vesicles may ...ear in the lumen.

...arlisle et al (1978), working with the isolated ...tric mucosa of the bullfrog *Rana catesbiana* ...gested that the theophylline-induced increase in ...sma membrane area is controlled by the cellular ...el of cyclic AMP These workers showed that ...ophylline in the presence of metiamide increased ...tric acid secretion and that the luminal surface ...the gland cells increased at the same time Theo-...ylline alone in the state of blockade acid secre-...n obtained either by anoxia of SCN increased ...luminal area of the oxyntic cells. By contrast, the present work, the oxyntopeptic cells revert to ...resting state in the presence of metiamide, ...pite the continuous injection of histamine ...ether these results are contradictory or not de-...ads on the mechanism of action of metiamide. If ...antagonist exerts its action by blocking ex-...*cellular H_2-receptors only* then it is under-...*ndable that the histamine-induced structural* ...anges revert to the ...esting state (as in the present ...ork) This effect was not obtained with theophyl-...e by Carlisle et al probably because theophylline ...ts at ...site beyond the receptor by inhibiting in-...cellular phosphodiesterase (Butcher et al 196..) ...is view is supported by Fromm et al (1975) who ...nd that histamine-induced but not theophylline ...db-cAMP-induced acid secretion in the isolated ...bit gastric mucosa, was blocked by metiamide. ...milar results have been reported by Sjöstrand et ...(1978), working with the isolated guineapig gas-...mucosa. By contrast Brown et al. (1975), using ...cosa from *Rana pipiens* presented evidence that ...tiamide acts at a step following cAMP Sinsay et ...(1978) in the same species found that the acid ...cretion stimulated by aminophylline or db-cAMP ...s antagonized by metiamide and concluded that metiamide exerts its action intracellularly The results of our study however are best explained by an effect of metiamide on extracellular receptors only

A high secretory activity of the gland cells is in accordance with the occurrence of special well developed organelles as well as with the close spatial arrangement of some of these organelles. Thus the well developed mitochondria occur close to the apical tubular system indicating the energy demand of the tubules as ion-secreting structures Well developed mitochondria also occur connected with the rough endoplasmic reticulum whose ribosomes might supply the adjacent vesicles with their contents of pro-enzymes As to the mitochondria the function of their granules has been discussed (Barnard & Afzelius 1972). Probably the granules form stores of calcium the concentration of which is suggested to be regulatory in acid secretion (Sachs et al. 1978). Possibly they represent stores of phospholipids and if so they are probably contributing to the abundance of membranes which aid in the secretion. All the ultrastructural data hence support the occurrence of a high secretory activity of these cells including secretion of acid as well as of proenzymes Ito & Schofield recently (1978) described how the parietal cells of mouse and cat change their morphology in response to stimulation and emphasized the importance of seeking a better understanding of how the cell structures are involved in the secretion. We believe that by studying different species and getting a broad phylogenetic knowledge it will prove possible to find the structural correlates of gastric secretion.

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ıanges in blood volume and extravascular water ntent in isolated perfused rat lungs durıng ntilation hypoxia

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AARSETH P BJERTNÆS L. & KARLSEN J Changes in blood volume and extravascular water content in isolated perfused rat lungs during ventilation hypoxia. Acta Physiol Scand 1980, 109 61–67 Received 20 Aug 1979 ISSN 0001-6772. Institute of Physiology University of Oslo Norway

Isolated rat lungs were perfused with homologous blood at constant volume inflow The effect of ventilation hypoxia on pulmonary vascular resistance preparation weight and reservoir volume (vascular capacitance) were studied In some periments also wet/dry eight ratio of the preparation was estimated (extravascular water content). There was no difference in this last parameter between hypoxic and normoxic lungs thus alveolar hypoxia had no effect on tissue water content as previously described in intact rats. With forward perfusion small and transient changes in either direction were seen in preparation weight and reservoir volume, even though inflow pressure exceeded 5 kPa during alveolar hypoxia. With backward perfusion marked weight increases were seen, and if inflow pressure in this situation was above 3.5 kPa, the weight change was irreversible thus indicating outward fluid filtration It is concluded that the vessels responding to alveolar hypoxia are located on the arterial side of the pulmonary vasculature

Key ords Microcirculation, pulmonary circulation, vascular resistance, lung water content, respiration, lung compliance

e pulmonary vasoconstrictor response to oxia acts t divert blood away from hypoxic a to nonhypoxic areas of lung (Hughes 1975) cording to Hauge (1969) alveolar hypoxia and not rmal hypoxemia is the main denominator of this oconstriction In concert with these observa-s, Bjertnæs Hauge & Torgrimsen (1978) report t equimolar concentrations of the inhalation sthetic halothane inhibits the vasoconstrictor ponse to hypoxia more effectively when ad-istered via the airways less via the pulmonary ery and least via the pulmonary eins. Although distinct anatomical location can be pointed out se authors suggest that the hypoxia-sensitive site located on the arterial side of the pulmonary sculature and functionally closer to the airways n to the responding blood vessels.

ontroversies exist, however about which vas-ar segment constrict upon hypoxia. Kato & ub (1966) have suggested that the constriction of all pulmonary arteries sufficiently explain the increase in pulmonary vascular resistance during ventilation hypoxia. Others have demonstrated increased venous resistance as well (Furnival Linden & Snow 1970 Hyman & Kadowitz 1975) Duke (1957) have proposed the increased resistance to be located to the capillaries Aarseth & Karlsen (1977) claimed that all the consecutive vascular segments are involved. The latter authors observed a marked reduction in blood volume (56 %) and in extravascular water content (33 %) during ventilation hypoxia in intact rats and postulated that changes in plasma osmolarity and/or nervous activity might be responsible.

The purpose of the present work has been twofold (1) to localize the vascular segments being involved in the pulmonary vasoconstrictor response to ventilation hypoxia and (2) to test whether this mechanism *per se* might be responsible for the reductions in blood volume and extravascular water content observed during ventilation hypoxia in lungs of intact animals

e 1 The effect of ventilation hypoxia on pulmonary tissue water content in forward perfused isolated ungs

est lungs, C control lungs

	Flow (ml/min)	Baseline P_{PA} (kPa)	ΔP_{PA} (kPa)	ΔP_{PA} (kPa)	Wet/dry weight	P_{TP} (kPa)	ΔP_{TP} (kPa)
T	[illegible]	1.81	2.84	3.35	5.60	1.66	0
C	13.0	1.81	2.45	0	6.01		
T	13.0	1.18	2.71	3.23	5.28	1.66	0
C	14.5	1.28	1.55	0	6.03		
T	[illegible]	1.68	1.94	2.32	5.38	1.44	0
C	13.0	1.03	[illegible]	0	5.19		
T	12.5	2.19	4.00	4.13	5.57	1.50	0
C	13.0	1.29	3.23	0	5.10		
T	14.0	1.81	2.84	3.87	4.82	0.98	0
C	16.0	2.45	2.58	0	5.91		
T	15.0	2.19	2.58	2.84	4.88	1.50	
C	14.0	2.19	1.68	0	4.45		
T	17.0	2.06	1.81	2.84	6.08		
C	15.0	1.55	2.97	0	4.67		

Pulmonary arterial pressor responses to hypoxia

recordings of the pulmonary arterial inflow pres- e and the corresponding weight changes of one the preparations are shown in Fig 2. Weight uctions of about 100 mg were observed during oxic pressor responses. When pulmonary ar- al pressure increased from 1.86 to 3.06 kPa sed by increased flow or from 1.81 to 3.74 kPa caused by the injection of 25 μg bradykinin the weight increased by 300 and 220 mg respectively. Flow reduction with corresponding pressure reduction resulted in decreased weight and increased reservoir volume. In another preparation weight increased 400 mg when inflow pressure was increased from 2.06 to 3.74 kPa by increased flow. Thus the

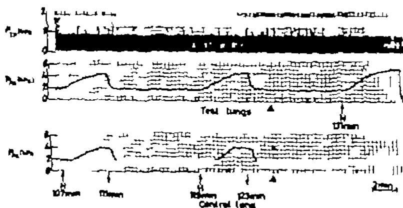


Fig 1. *Upper curves*: Transpulmonary pressure (P_{TP}) and pulmonary artery pressure (P_{PA}) of the test lungs. *Lower curve*: Pulmonary artery pressure of the control lungs. Typical responses to 4 min periods of ventilation hypoxia are elicited in both preparations. [14]C-labelled erythrocytes in [125]I-albumin was added to the blood reservoir of each preparation before the last hypoxic period of the test lungs (▲). The closed circles indicate removal and rapid freezing of both preparations.

METHODS

Wistar rats weighing between 200 and 250 g were used The operation procedure has been described previously (Bjertnaes 1977) The heart lung preparation was removed from the chest and the pulmonary artery and the left atrium were cannulated The inflow cannula was con nected to a Statham P 23 Db transducer and an airtrap

The lungs were perfused with heparinized (10 I U./ml) homologous blood by means of a roller pump the blood draining back to a thermostated reservoir Inflow pressure was monitored on a Sanborn Model 2200 six channel recorder and flow was set to give an initial inflow pressure of 1.0–1.5 kPa. Since outflow pressure and flow were kept constant in each experiment changes in pulmonary vascular resistance were directly reflected as changes in pulmonary inflow pressure Ventilation which was usually performed with a Starling "Ideal" pump started with 2–3 gentle hyperinflations to a pressure of 2 kPa and the volume was then set to give an initial inflation pressure not exceeding 1 kPa The frequency was 70 inflations per min. The end-expiratory pressure was 0 15–0.2 kPa. Transpulmonary pressure (P_{TP}) was continuously recorded The ventilation gas was 5% CO_2 in air for periods of 8 min interposed by repeated 4 min periods of ventilation with a gas mixture containing 2% O_2 5% CO_2 and 93% N During these periods the perfusate was equilibrated with the P_{O_2} of the ventilation gas A vasoconstrictor response was usually evoked after 2–5 such hypoxic periods

The lung preparations were suspended underneath a force transducer Model FTA 100-1 (Hewlett Packard) and the weight changes were continuously registered on the Sanborn recorder In some experiments effluent blood from the lung preparation was drained via a 1 ml syringe mounted on the top of the blood reservoir Thus reservoir volume changes down to 0 05 ml could be observed con comitantly with the registration of weight changes

Three series of experiments were performed

Series I In 7 expts two lung preparations were for ward perfused simultaneously but independently The preparations were given the same handling and perfusions were started only 5–10 min apart When the pulmonary pressor response had reached a certain magnitude in both lung pairs one of them was chosen by random to be the test lung preparation whereas the other served as control A suspension of ⁵¹Cr labelled rat erythrocytes in ¹²⁵I-serum albumin solution (Institutt for atomenergi Kjeller Norway) was then added to the perfusates giving an activity of about 80 000 c p.m of each isotope per ml After a mixing period of 6 min, hypoxia was given to the test lungs. At the top of the hypoxic response both preparations were rapidly removed and frozen in liquid nitrogen From the isotope content of the lung preparations and perfusate samples the pulmonary blood content was calculated thus allowing estimation of the wet/dry weight ratio of the lung tissue proper as previously described (Aarseth & Bo 1972) In each experiment the tissue water content could be compared between the hypoxic and the normoxic preparation

Series II Forward perfusion of 5 single preparations Preparation weight and reservoir volume were followed in all experiments during periods with alveolar hypoxia In preparations these parameters were followed i[illegible] periods with increased flow and thereby increased [illegible] lar pressure In 1 preparation vascular resistance [illegible] flow pressure were increased by injection of bradykinin (Sandoz A G)

Series III Backward perfusion of 7 single prepar[illegible] Preparation weight changes were recorded in all [illegible] ments and reservoir volume changes were registered [illegible] expts during periods of alveolar hypoxia. In 4 pre[illegible] tions the tissue water content was finally evaluated [illegible] the group 1 expts Three of these preparations we[illegible] moved during hypoxia one during normoxia. For [illegible] cal reasons it was not possible to include controls [illegible] lel perfusion

For statistical evaluation of the results, Wilcox[illegible] sample test (two-sided) was used

RESULTS

During the initial part of the perfusion [illegible] with ventilation hypoxia induced no or only [illegible] small pressor responses The number of hyp[illegible] periods varied between ? and 8 in different prep[illegible] tions before hypoxic pressor responses of [illegible] than 1.3 kPa were obtained

Series I Recordings from one expt. are de[illegible] strated in Fig 1 Increasing pressor responses [illegible] repeated periods of ventilation hypoxia were [illegible] taneously evoked in both lung pairs [illegible] changes in P_{TP}, indicating that vascular [illegible] were not influenced (Hauge Bo & Waaler 197[illegible] 7 expts listed in Table 1 median inflow pressure [illegible] the test lung preparation was 1 8 kPa (range [illegible] 2 19 kPa) with a median flow of 12.5 ml/min (r[illegible] 12–17 ml/min) The corresponding values for control lung preparations were 1 3 kPa (range [illegible] 2 45 kPa) and 14 ml/min (range 13–16 ml/min) [illegible] ing the last hypoxic period the median pres[illegible] increase in the test lung preparation was 3.23 [illegible] (range ? 32–4 13 kPa) No change in P_{TP} was [illegible] served in any experiment The wet/dry w[illegible] ratios showed no difference between the test [illegible] and the control lungs and thus no effect of ve[illegible] tion hypoxia on tissue water content was detect[illegible]

Series II In 5 preparations with forward pe[illegible] sion only small and inconstant weight changes [illegible] observed during periods with ventilation hypo[illegible] which gave pressor responses between 1.3 and [illegible] kPa The observed weight changes were parall[illegible] by opposite changes in the reservoir volume T[illegible] complete results including flow baseline pul[illegible] nary arterial inflow pressure pressor response[illegible] hypoxia and the concomitant weight and vol[illegible] changes are presented in Table

le 3 The effect of ntilation hypoxia on tldry ight and on changes in preparation weight (ΔW) reservoir blood olume (ΔV) i isolated rat lu gs during back ard perfusion

Expt	Flow ml min	Baseline P_{PA} (kPa)	ΔP_{PA} (kPa)	ΔW (mg)	ΔV (µl)	P_{TP} (kPa)	ΔP_{TP} (kPa)	% change	% reversibility ΔP_{TP}	Wet/dry Weight
i	13	1 81	0 76	125		1.37	0 04	3	100	
		1.55	0 45	0		-35	0 04	3	100	
		1 68	1.55	250		1 47	0 12	8	100	
		1.55	1.81	0		1 49	0 1	8	100	
		1.55	3 48			1.51	0 10	7	100	
		97	3_			1 70	0 06	4	No	8 87
	L	1 68	0 90	875		1.32	0 08	9	75	
	3	0 90	1.55	925	–	1.50	0.16	10	100	
	3	1 03	1.55	1 200		1.57	0 18	11	100	
	3	1 03	1 68	1 300		1.65	0.1	7	No	5.62
	16	1 55	1 16	1 000		1 72	0.18	10	50	
		1 29	0 39	200		1.80	0 08	4	100	
		1 79	0 52	290		1.80	0.08	4	100	
		1.29	19	1 500	–	1.80	0.1	7	100	
		1 4	3 35	3 700		1.80	0 12	7	No	9.32
	7	1 94	0	0					–	
		1 55	1 13	0						
		1 55	0.77	290						
		1 47	1.55	500						
		1 42	06	900		1.29	0.20	15	80	
		1 42	2 45	600		1.37	0 20	14	N	6.19
	10	1 16	1 16	800	650					
		1.29	1 16	750	600					
		1 29	1 42	850	700					
		1 29	06	1 050	1 000				–	
		1 42	06	1 000	900	–	–		–	
	10	1 29	1 42	700	700	1 41	0 06	4	100	
		1 42	2.19	1 175	1 050	1 14	0.20	17	40	
	15	1 29	1 55	572	400	0 94	0 18	19	50	
		1 16	-32	803	700	1.29	0 16	1	100	
		1 29	2 97	1 034	900	1 97	0 14	10	100	
		1 29	3 23	1 100	1 000	1 47	0.10	7	100	
		1.29	3 74	1 760	1 550*	1 57	0.39	25	No	

lveolar flooding
min entilation hypoxia, 4 min in all other hypoxic periods
and ΔP_{TP} are increases in pulmonary enous utflow pressure and transpulmonary pressure respectively

maximum reversible increments in P_{TP} (ΔP_{TP}) ng hypoxia (Table 3) was 0 12 kPa (range 0 06– 8 kPa) starting from a median P_{TP} of 1 49 kPa nge 1.29–1.80 kPa)

SCUSSION

es pulmonary vasoconstriction in response to oxia per se cause a reduction in blood volume 3 tissue water content of the lungs? We ha e ted this hypothesis by the use of two indepen- t experimental procedures. The double isotope hnique (Aarseth & Bo 1977) showed no differ ce in wet/dry weight ratio during forward perfu sion between the test lungs and the control lungs (series I) Since the lungs had to be removed from the experimental upset in order to be frozen in liquid nitrogen, redistribution of minute amounts of blood from the lungs into the outflow tubings might have taken place. A second series of expts. there fore was carried out (series II) in which changes in preparation weight and reservoir volume could be continuously registered. In these preparations the weight changes either were small absent or biphasic consisting of a weight reduction followed by an increase The weight changes were accompanied by volume changes in the opposite direction but of a corresponding magnitude, which pro ed

Table 2 *Changes in preparation weight (ΔW) and reservoir blood volume (ΔV) during ventilation hypoxia in forward perfused isolated rat lungs*

Expt. no	Flow (ml/min)	Baseline P (kPa)	ΔP_P (kPa)	ΔW (mg)	ΔV (μl)
8	1?	19	2 19	−100	+100
9	10	2.32	1.29	0	0
10	1	2.58	1 81	0	0
11	1?	2 06	2 45	−50/ +100	+50/ −100
12	11	1.81	2 45	+30	−30

Pulmonary arterial pressure



Fig 2 Simultaneous recordings of pulmonary a[rterial] pressure and weight changes in forward perfused is[olated] rat lungs Effects on lung weight of pulmonary a[rterial] pressure increases in response to hypoxia, to incr[eased] flow and to injection of bradykinin are demonstrated

weight changes seen during hypoxia are not only passively due to pressure changes per se

Series III 7 preparations were retrogradely per fused through the left atrial cannula. Tracings of the transpulmonary pressure (P_{TP}) the weight changes (Δw) and the pulmonary venous inflow pressure (P_{PV}) of one representative expt (No 19) are shown in Fig 3 The hypoxic pressor responses during backward perfusion were comparable with those seen during forward perfusion However during backward perfusion concomitant increases in transpulmonary pressure and in the preparation weight were seen during the hypoxic periods P_{TP} changes were completely reversible upon cessation of ventilation hypoxia. The initial weight was not regained however and the remaining weight gain was larger with increasing pressor responses When a certain response magnitude was exceeded pul monary edema developed and neither of [the] changes returned to baseline (last sequence Fi[g] The same weight response was seen if flo[w] thereby transmural pressure was reduced (exp[t] 14)

The complete results from this series of e[xpts] are listed in Table 3 The wet/dry weight ratio [was] examined in 4 of the preparations In 2 preparat[ions] (no 13 and 15) with pressor responses of 3 ... 3 35 kPa respectively the wet/dry weight r[atios] were markedly increased In 2 other preparat[ions] (no 14 and 16) modest pressor responses [were] seen and in these preparations normal tissue [water] content was found In the 3 expts (no 17 ... 19) in which volume changes were measured weight increases during pressor responses [to] hypoxia were paralleled by corresponding re[duc]tions in the reservoir volume The median val[ue]

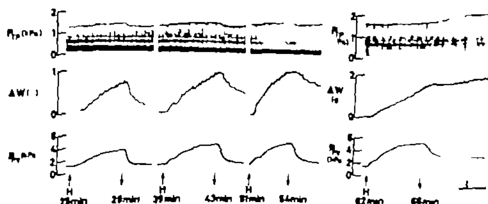


Fig 3 Simultaneous recordings of transpulmonary pressure (P_{TP}) lung weight change (ΔW) and pulmonary venous pressure (P_{PV}) during backward perfusion of an isolated rat lung preparation (exp no 19) The arrows indicate start and termination of ventilation hypoxia (F_{IO_2} 0 02) Time is given in min from start of perfusion The reversibility of weight changes decreased with increasing pressure responses The changes in P_{TP} became irreversible first when alveolar flooding occurred (last sequence)

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that the fluid volume of the lungs really changed The rapid off-response upon reversion of the pressor response to hypoxia shown in Fig 2 also points to an intravascular volume change The change in tissue water content observed in series I and the changes in fluid volume observed in series II were randomly distributed and thus no effect of hypoxia on lung blood volume and tissue water content could be demonstrated in isolated rat lungs These findings contrast the marked reductions reported from experiments in intact animals by Aarseth & Karlsen (1977) The discrepancy between the observations in the two studies support the hypothesis that changes in plasma osmolarity to a great extent might have been responsible for the lung tissue dehydration in situ although nervous mechanisms have not been ruled out

A problem of considerable physiological interest has been to localize the effector site of the pulmonary vasoconstrictor response to hypoxia Intuitively the most logical way of producing high altitude pulmonary edema would be to increase the pulmonary capillary pressure Actually there has been presented evidence for increased pulmonary venous resistance during hypoxia in the dog (Rivera Estrada et al 1958) Furnival Linden & Snow 1970) and in the lamb (Hyman & Kadowitz 1975) Severinghaus (1971) however suggests that arterial leakage takes place during hypoxia whereas Hultgren & Grover (1968) have experience for uneven distribution of vasoconstriction which might lead to relative overperfusion and edema development in some regions of the lungs and underperfusion in others

The blood content of intact rat lungs has been calculated to be about 1 ml (Aarseth & Karlsen 1977) When the inflow pressure in backward perfused rat lung preparations (series III) increased to a level below about 3 5 kPa during hypoxia lung weights increased reversibly with 100–150% of this amount indicating vascular distension upstreams to an increased resistance Simultaneous with the rise in inflow pressure compliance decreased reversibly by maximum 12% (expt no 19) Such a reversible compliance reduction has also been shown to be associated with pulmonary blood volume increments during left atrial pressure elevations in the cat (Hauge Bo & Aarseth 1977) and in the isolated rabbit lung preparation (Hauge Bo & Waaler 1975) When however inflow pressure during backward perfusion rised to a level above about 3 5 kPa in response to hypoxia frank pulmonary e[illegible] developed (Expt no 13 15 and 19) and the re[illegible]tions in compliance became irreversible ([illegible] sequence Fig 3) In contrast no significant filtration occurred during forward perfusion [illegible] when inflow pressure exceeded 5 kPa during [illegible]tilation hypoxia (Table 1)

In conclusion the considerable weight [illegible] during pressor responses to hypoxia in back[illegible] perfused lungs including outward filtration [illegible] the plasma colloid osmotic pressure [illegible] exceeded the capillary transmural pressure and the ab[illegible] of these changes during forward perfusion str[illegible] suggests that vasoconstriction in response [illegible] hypoxia is located to precapillary vessels Th[illegible] well with the hypothesis that the receptor are[illegible] hypoxia induced vasoconstriction is situated on arterial side of the pulmonary vasculature (B[illegible]næs Hauge & Torgrimsen 1978)

Further experiments however must be car[illegible] out to investigate whether enhanced plasma o[illegible]larity or increased sympathetic nervous activ[illegible] responsible for the reductions in blood volume tissue water content observed during hypoxia intact rat lungs

The skilled technical assistance of Ms Karin Lage[illegible] greatly appreciated

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ɔld post ejaculatory inhibitory effect of seminal plasma .sperm nuclear chromatin decondensation ability in man

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The stability of the nuclear chromatin in human spermatozoa soon after ejaculation was studied by exposing the cells to sodium dodecyl sulphate (SDS) one to 20 min after ejaculation. Semen samples ere obtained both from men with apparently normal, and from men ith impaired prostatic secretion (=subnormal seminal plasma [Zn]). The sperm nuclear resistance to decondensation in SDS increased in both groups during the first 15 min after ejaculation but was significantly lower in the semen samples with subnormal [Zn] This fast post-ejaculatory increment in sperm SDS resistance was significantly re duced by 5-fold saline dilution of the semen at the time of ejaculation. It is discussed if the observed stabilization with time was illusory and that prostatic component instead counteracted an intrinsic nuclear chromatin decondensation (NCD) process initiated by SDS derangement of spermatozoal membranes.

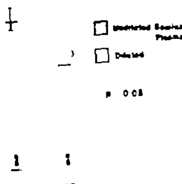
Key ords. Spermatozoa, human, chromatin stability seminal plasma, prostate nuclear chromatin decondensation, zinc

ɑmmalian spermatozoal chromatin is apparent- tabilized by the formation of disulphide) bridges (Miescher 1878 cf Bedford 1975) ɪport for this concept is the observation by Bed- l and co-workers that spermatozoal chromatin ɓility increases during the epididymal passage also that this increase is concomitant with a rease of free thiol (SH) groups in the nucleus lvin & Bedford 1970 1971 Bedford, Bent & ın 1973 Calvin, Y & Bedford 1973).

ɦe elocity of nuclear swelling during a com ed treatment with dithiothreitol (DTT) and lium dodecyl sulphate (SDS) is one way to assess ɤm chromatin stability (Calvin & Bedford 1971) cording to these authors such a treatment causes econdensation of spermatozoal nuclear chroma- ɩince DTT disrupts S-S bonds (Cleland 1964) l SDS dissol es not S-S stabilized spermatozoal uctures (e.g. plasma membranes) Bedford et al 973) observed that a rather high percentage of ɤmatozoa from some men also swelled in SDS ɪne when exposed for 60 min. The latter observa- tion was recently confirmed (Kvist & Eliasson 1980) It was then also noted that SDS sensitive spermatozoa occurred more frequently in semen samples with a subnormal zinc/fructose ratio and that brief (5 min) exposure of such spermatozoa to exogenous seminal plasma with normal level of zinc and other prostatic components ncreased their resistance to SDS Hence these observations indicated that some seminal plasma component of prostatic origin either stabilized spermatozoal chromatin or preserved its stability It was therefore of interest to study if such seminal plasma components influenced spermatozoal chromatin stability already within 20 min after ejaculation

MATERIAL AND METHODS

Semen specimens were obtained from 11 volunteers All specimens were collected by masturbation after 3–5 days of continence 8 donors ere known to hav normal composition of the seminal plasma as judged by the biochemical markers for prostatic secretion ($Zn > 1.2$ mM $Mg > 2.9$ mM and acid phosphatase activity >6 9 mKat/l), and for



Reduction of post-ejaculatory human sperm nuc resistance to SDS (sodium dodecyl sulphate) after d saline (BSS) dilution of the semen at the time of ulation when the spermatozoa subsequently were sed to SDS ($P<0.05$)

groups had higher resistance to SDS when served in seminal plasma from one to 15 min r ejaculation (P-values for both groups <0.01). wever the spermatozoa ejaculated in seminal sma with abnormal prostatic contribution oup I) were less table than spermatozoa in up II both at one ($P<0.05$) and at 15 min <0.01) after ejaculation. In fact the latter group) revealed almost maximal spermatozoal chroma- stability (mean 97%) after 15 min in seminal sma, whereas the corresponding figure for group as 66%

fect of post jaculatory milieu

e effect of the su ounding milieu at the time of aculation wa investigated in a series of experi- ents where three donors delivered 2 semen sam- es each and a fourth donor delivered 4 semen mples (e $N=10$). Half of the samples from each nor served as controls and were allowed to quefy for 20 min after ejaculation in their own minal plasma. An aliquot of each of these samples as then subjected to SDS treatment. Five other mples were ejaculated in boxes containing four olumes of 37°C BSS and at once mixed with the SS After 20 min aliquot of these samples were lso subjected to SDS treatment. The spermatozoa xposed to the BSS diluted seminal plasma revealed significantly lower resistance to SDS than sperma- ozoa in the undiluted seminal plasma ($P<0.05$) Fig).

DISCUSSION

The effect of anionic detergents like SDS on sper matozoal structures has to be considered before discussing the value of the SDS test as an index of the prevailing sperm nuclear chromatin stability Apparently SDS per se does not to any appreciable extent cause the disruption of S-S bonds reported necessary to induce nuclear chromatin decondensa- tion However SDS in a few minutes makes possi- ble the complete swelling of spermatozoa which at the onset of SDS exposure contain nuclear chroma- tin not fully stabilized by S-S bonds (Bedford Cal vin & Cooper 1973) Nonetheless, in a previous study it was observed that a high proportion of ejaculated spermatozoa from some men underwent progressive swelling during 60 min of SDS exposure (Kvist & Eliasson 1980) It indicates that a process involving gradual disruption of essential chemical bonds in the chromatin (e g. S-S bonds) under cer tain circumstances may take place in the sper matozoa, and that this process perhaps is initiated by the SDS-induced derangement of spermatozoal membrane structures In the study by Kvist & Eliasson (1980) it was also found that the highest proportion of spermatozoa swelling during 60 min in SDS was present in semen with subnormal zinc/ fructose ratio and that brief exposure of such un stable spermatozoa to seminal plasma with normal prostatic secretory contribution counteracted swel- ling in SDS

The results of the present tudy provide further support for the idea that a factor in seminal plasma of prostatic origin has the ability to protect ejacu- lated spermatozoa against swelling in SDS and suggest that this protection is rapidly established Thus, a considerable increase of the spermatozoal chromatin resistance against swelling in SDS took place already during the first 15 min after ejacula- tion (Fig. 1*A*) and semen from men with apparently normal prostatic secretion contained a much larger proportion of SDS resistant spermatozoa than did semen from men with impaired prostatic function at one as well as at 15 min after ejaculation (group II vs group I in Fig. 1*B*) Further evidence that nor mal concentrations of some component in the seminal plasma are needed to induce high swelling resistance in SDS is provided by the present obser ation that immediate post-ejaculatory saline dilu- tion of the semen significantly increased the percen tage of spermatozoa swelling during SDS exposure



Fig 1 Increase in human sperm nuclear resistance to decondensation in sodium dodecyl sulphate (SDS) during the first 15 min after ejaculation. *A* = 14 samples from 7 donors both from men with apparently normal prostatic secretion and from men with impaired prostatic function ($P<0.001$) *B* = A subdivision of the material into a low zinc group (I) (zinc conc 0.4–0.7 mM (I)) and a normal zinc group (>1.2 mM (II)) Significances for differences Group I 1 min vs. 15 min $P<0.01$ Group II 1 min vs. 15 min $P<0.01$ Group I vs group II 1 min $P<0.05$ Group I vs. group II 15 min $P<0.01$

seminal vesicular secretion (fructose >6.7 mM) (cf Eliasson 1975) Three donors were known to have prostatic hyposecretion (i.e subnormal concentrations of prostatic components) Zinc and magnesium in seminal plasma were measured with an atomic absorption spectrophotometer (cf Eliasson & Lindholmer 1971 1972) Acid phosphatase activity was determined according to Sigma Technical Bulletin No 104 (1975) and fructose concentration with a colorimetric technique (Karvonen & Malm 1956) as described by Eliasson (1965)

To reduce the possible influence of seminal plasma components on the nuclear chromatin stability during the first 20 min after ejaculation (i.e during the gel-phase before complete liquefaction), semen was in some experiments diluted at the time of ejaculation Dilution was obtained by collecting the ejaculate in a plastic box containing 37°C buffered salt solution (BSS) to the amount of four times the expected semen volume The composition of BSS was (mM) NaCl 134 KCl 5 HCl 4 TRIS 37 pH 8.0

Spermatozoal nuclear chromatin stability was assessed as the degree of nuclear swelling after the spermatozoa had been exposed for 60 min to 1% of sodium dodecyl sulphate (SDS in 0.05 borate buffer pH 9.0)

One volume of native or diluted semen was mixed with nine volumes of the SDS solution and the reaction was stopped by adding an equal amount of 2.5% glutaraldehyde in 0.05 M borate buffer pH 9.0 Nuclear swelling was assessed with phase contrast microscopy (500 ×) and the spermatozoa were classified as stable (= not swollen) and unstable (moderately and grossly swollen) (Kvist & Eliasson 1980).

For descriptive statistics mean and range are given for percentage stable (= non-swelling) spermatozoa and seminal plasma zinc concentrations Analytical statistics were performed by using the Randomization Test for Matched Pairs and the Mann Whitney U Test for independent groups (cf Siegel 1956)

RESULTS

Effect of postejaculatory time

Fourteen semen specimens from seven donors were used to find out if spermatozoal resistance to SDS increased already during the first 15 min after ejaculation Three of the donors (7 specimens) had seminal plasma zinc values between 0.4–0.7 mM. The seminal plasma zinc values of the other donors (7 specimens) were 1.4–3.5 mM Aliquots of each semen specimen were exposed to SDS at one and 15 min after ejaculation As shown in Fig. 1A spermatozoa which had remained in their seminal plasma for 15 min had considerably higher resistance to SDS than those exposed to SDS at 1 min after ejaculation ($P<0.001$) At the same time the range of percentage stable spermatozoa was narrower

A subdivision of the material into two groups with reference to subnormal (I) and normal (II) seminal plasma zinc values (Fig 1B) revealed that

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…versible inhibition of nuclear chromatin …condensation (NCD) ability of human spermatozoa …duced by prostatic fluid

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KVIST U Reversible inhibition of nuclear chromatin decondensation (NCD) ability of human spermatozoa induced by prostatic fluid Acta Physiol Scand 1980 109 73–78. Received 30 Aug. 1979 ISSN 0001-6772. Reproductive Physiology Unit, Department of Physiology Karolinska Institutet, Stockholm, Sweden.

In semen from donors with adequate secretory function of the prostate spermatozoa in the first ("prostatic") portion of the ejaculate were more resistant to nuclear swelling in sodium dodecyl sulphate (SDS) than spermatozoa from the second ("vesicular") portion. N such difference as revealed by donor with severely impaired prostatic function. This demonstrates that some sperm nuclear chromatin stabilizing factor(s) is present in normal prostatic fluid. The chromatin stabilizing factor( ) could largely be removed by washing the spermatozoa in saline containing albumin. Spermatozoa sensitized to SDS in this manner *regained* their SDS resistance upon exposure to normal (zinc-rich) prostatic fluid" Such exposure also induced high degree of resistance in natively SDS sensitive spermatozoa. The possibility is discussed that zinc of prostatic or other origin reversibly inhibits a nuclear chromatin decondensation ability (NCD-ability) It is suggested that such mechanism may be of essential importance for male genome transfer

Key words Spermatozoa, human, chromatin stability seminal plasma, prostate, nuclear chromatin decondensation, zinc

…man semen sometimes contains a relatively high …centage of spermatozoa reacting with nuclear …omatin decondensation (i.e. swelling) when …osed for 60 min to the anionic detergent sodium …decyl sulphate (SDS) (Bedford, Bent & Calvin …; Kvist & Eliasson 1980) Kvist & Eliasson …80) also found that semen samples with an abso… …ly or relatively low content of prostatic markers … subnormal zinc/fructose ratio) contained sig… …cantly more spermatozoa swelling in SDS than …en samples from men with apparently normal …static function Furthermore spermatozoa from …an with an impaired prostatic function became …siderably more resistant to SDS when they were …ally exposed to seminal plasma of normal com…sition Further evidence that a seminal plas… component of prostatic origin inhibits nuclear …omatin decondensation and thus contributes to … preservation of post-ejaculatory chromatin sta…lity has subsequently been presented (Kvist …80) and it was suggested that prostatic component (possibly zinc) may normally counteract an intrinsic nuclear chromatin decondensation (NCD) process initiated by a SDS derangement of spermatozoal membranes.

The split ejaculate technique (Lundquist 1949 Eliasson 1965) provides a tool to separate semen fractions with predominantly prostatic contribution to the seminal plasma from fractions in which the plasma mainly consists of fluid from the seminal vesicles. Therefore with the split ejaculate technique it has now been possible to obtain further support for the concepts that a factor of prostatic origin contributes to preserve the integrity of human spermatozoal chromatin by reversibly inhibiting of NCD-ability

MATERIAL AND METHODS

Semen specimens were obtained from men referred to the laboratory for barren union, and from volunteers (mainly medical students). All semen samples were collected by masturbation after 3–5 days of continence Split ejaculates

(Fig ?) The nature of the active components in the seminal plasma remains to be elucidated However the observed co-existence of a reduced spermatozoal resistance to SDS and a subnormal seminal plasma zinc concentration and the preliminary observations that zinc could increase sperm SDS-resistance (Eliasson & Kvist 1976 Kvist & Eliasson 1978) makes zinc worth further consideration

Appreciable amounts of zinc is generally incorporated into mammalian spermatozoa before they reach the prostate but there are also indications that zinc in human and dog spermatozoa can be derived from the prostate (Janick Leitz & Whitmore 1971 Saito Leitz & Bush 1967) Thus zinc may contribute *to establish a resistance to SDS in spermatozoa* prior to ejaculation

The possibility that zinc or some other component in seminal plasma acts by inhibiting an intrinsic sperm nuclear chromatin decondensation (sperm NCD) process triggered by a SDS induced derangement of spermatozoal membranes is suggested and warrants further investigations

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...eversible inhibition of nuclear chromatin ...econdensation (NCD) ability of human spermatozoa ...nduced by prostatic fluid

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KVIST U Reversible inhibition of nuclear chromatin decondensation (NCD) ability of human spermatozoa induced by prostatic fluid Acta Physiol Scand 1980, 109 73–78. Received 30 Aug. 1979 ISSN 0001-6772. Reproductive Physiology Unit Department of Physiology Karolinska Institutet, Stockholm, Sweden.

In semen from donors with adequate secretory function of the prostate spermatozoa in the first ("prostatic") portion of the ejaculate were more resistant to nuclear swelling in sodium dodecyl sulphate (SDS) than spermatozoa from the second ("vesicular") portion. No such difference was revealed by a donor with severely impaired prostatic function. This demonstrates that some sperm nuclear chromatin stabilizing factor(s) is present in normal prostatic fluid. The chromatin stabilizing factor( ) could largely be removed by washing the spermatozoa in saline containing albumin. Spermatozoa sensitized to SDS in this manner regained their SDS resistance upon exposure to normal (zinc-rich) prostatic fluid" Such exposure also induced high degree of resistance in natively SDS sensitive spermatozoa. The possibility is discussed that zinc of prostatic or other origin reversibly inhibits a nuclear chromatin decondensation ability (NCD-ability). It is suggested that such a mechanism may be of essential importance for male genome transfer

Key ords Spermatozoa, human chromatin stability seminal plasma, prostate nuclear chromatin decondensation, zinc

...uman semen sometimes contains a relatively high ...rcentage of spermatozoa reacting with nuclear ...romatin decondensation (i.e. swelling) when ...posed for 60 min to the anionic detergent sodium ...decyl sulphate (SDS) (Bedford, Bent & Calvin ...73 K ist & Eliasson 1980) Kvist & Eliasson ...980) also found that semen samples with an abso...ely or relatively low content of prostatic markers ...e subnormal zinc/fructose ratio) contained sig...ficantly more spermatozoa swelling in SDS than ...d men samples from men with apparently normal ...ostatic function Furthermore spermatozoa from ...men with an impaired prostatic function became ...nsiderably more resistant to SDS when they were ...iefly exposed to seminal plasma of normal com...sition Further evidence that a seminal plas... component of prostatic origin inhibits nuclear ...romatin decondensation and thus contributes to ... preservation of post-ejaculatory chromatin sta...ility has subsequently been presented (Kvist ...80) and it was suggested that a prostatic compo-nent (possibly zinc) may normally counteract an intrinsic nuclear chromatin decondensation (NCD) process initiated by a SDS derangement of spermatozoal membranes.

The split ejaculate technique (Lundquist 1949 Eliasson 1965) provides a tool to separate semen fractions with predominantly prostatic contribution to the seminal plasma from fractions in which the plasma mainly consists of fluid from the seminal vesicles. Therefore with the split ejaculate technique it has now been possible to obtain further support for the concepts that a factor of prostatic origin contributes to preserve the integrity of human permatozoal chromatin by reversibly inhibiting of NCD-ability

MATERIAL AND METHODS

Semen specimens were obtained from men eferred to the laboratory for barren nation, and from volunteers (mainly medical students). All semen samples were collected by masturbation after 3–5 days of continence. Split ejaculates

Table I *Percentage of SDS resistant spermatozoa and zinc concentration (mM) in prostatic portion and vesicular portion (II) of split-ejaculates from 5 donors with apparently normal prostatic func (Group A) and one donor (5 samples) with impaired prostatic function (Group B). Mean and range*

Fraction	Percentage stable spermatozoa			Portion zinc concentration		
	I	II	Δ	I	II	Δ
Group A						
Mean	77	60	16	2.9	0 7	2*
Range	(61–99)	(37–83)	(12–24)	(1.5–5 7)	(0 1 1 6)	(1 1–4 1)
Group B						
Mean	58	61	3	0.5	0.4	0.1
Range	(52–63)	(56–66)	(–5–7)	(0 4–0.7)	(0.3–0 6)	(0.0–0.3)

$P<0.05$
Not significant.

were generally collected in 6 fractions which afterwards were pooled into two portions (Lundquist 1949) Portion I consisted of the 3 first fractions (seminal plasma with mainly prostatic contribution) and remaining fractions (with high seminal vesicular contribution) were pooled as Portion II. Occasionally experienced donors were instructed to collect the ejaculate directly in the two portions mentioned. This proceeding was for example used in experiments involving an immediate post-ejaculatory five-fold saline dilution of the semen according to the technique previously described (Kvist 1980)

Spermatozoa were studied either in aliquots of the semen portions or after having been concentrated by mild centrifugation (400 g for 15 min) followed by removal of the supernatant. Washed spermatozoa were prepared by washing the centrifugation pellets twice in either a magnesium free buffered salt solution (BSS) or in BSS containing 4% human albumin (BSSA) The composition of BSS was (mM) NaCl 123 KCl 5 HCl 4 TRIS 37 (pH 8.0) Seminal plasma from Portion I ("prostatic fluid") and from Portion II ("vesicular gland fluid") were obtained by centrifugation (2 500 g for 20 min) of the two portions and collection of the supernatants

Nuclear chromatin stability was assessed from the degree of sperm head swelling after 60 min exposure to 1% sodium dodecyl sulphate (SDS) in 0 05 M sodium borate buffer (pH 9 0) One volume of semen or sperm suspension was mixed with 9 volumes of the SDS solution and the reaction was stopped by adding the equal amount of 2.5% glutaraldehyde in 0 05 sodium borate buffer (pH 9 0) The degree of nuclear swelling was then semi-quantitatively evaluated by phase contrast microscopy (500×) and scored as previously described (Kvist & Eliasson 1980)

The secretory contribution of the prostate was assessed by measuring the seminal plasma zinc and magnesium concentrations with an atomic absorption spectrophotometer (cf Eliasson & Lindholmer 1971 1972) and its acid phosphatase activity (cf Sigma Technical Bulletin No 104). An index of the vesicular gland secretory contribution was obtained by colorimetric determination of the seminal plasma fructose concentration (Karvonen & Malm 1955) as described by Eliasson (1965)

For descriptive statistics mean and range is given for determined percentage of SDS resistant spermatozoa and for the chemical variables. Analytical statistics was performed with the Randomization Test for Matched P (cf Siegel 1956)

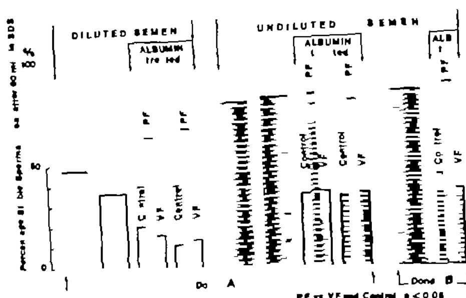
RESULTS

A Albumin induction of sperm NCD-ability

Spermatozoa from 6 semen samples (obtained fr different donors) were subjected to washing 3 to after ejaculation One half of each spermatozoa population was washed in BSS and the other half were washed in BSSA The spermatozoa were th subjected to SDS exposure for 60 min Those sa ples washed in BSS contained on the average 9 stable spermatozoa while those washed in BS had a mean percentage stable spermatozoa of 8 (mean difference 8 range 0–18 $P<0.05$) However in a corresponding series of experiments (5 se samples from different donors) the spermato were washed in BSS or BSSA already 15 min a ejaculation and the differences were consider greater The mean percentage stable spermato of aliquots washed in BSS was 56 and of th washed in BSSA 35 (mean difference 21 range 1 39 $P<0.05$)

B Difference in sperm NCD-ability between split-ejaculate portions

Five donors with previously observed normal co position of the seminal plasma and one donor w



1 Restoration of nuclear chromatin stability of experimentally "destabilized" spermatozoa by the exposure to ...dogenous "prostatic fluid" (PF). Endogenous "vesicular fluid" (VF) lacks this effect. Nuclear destabilization was ...duced by immediate post-ejaculatory saline dilution of the semen and/or washing in albumin containing saline 20 min ...er ejaculation.
...ntrol albumin washed and albumin exposed spermatozoa
...S Sodium dodecyl sulphate
...th donors had apparently normal prostatic function. For further details see text.

...diagnosed impairment of the prostatic function ...livered split-ejaculates as portions I and II. The 5 ...normal" donors provided one sample each, ...hereas the donor with the impaired prostatic func...on delivered 5 samples. Within 30 min after ejacu...tion the spermatozoa in each portion were sub...cted to SDS exposure for 60 min.

As shown in Table 1 the samples from the "nor...al" donors contained a significantly higher per...ntage of SDS resistant spermatozoa in the zinc ...ch portion I ("prostate dominated portion") than ... the zinc-poor portion II ("vesicular fluid domi...ated portion"). In contrast, the semen samples ...om the donor with impaired prostatic function did ...ot reveal any difference in the percentage SDS ...resistant spermatozoa between portions I and II. ...ere the zinc concentration in portion I was almost ...low as in portion II (Table 1).

Effect of "prostatic fluid" on sperm NCD-ability

Experimentally induced SDS sensitivity. The ef...ct of "prostatic fluid" vs. "vesicular fluid" on the ...hromatin decondensation ability was studied on experimentally SDS-sensitized spermatozoa. Increased SDS-sensitivity was obtained by an immediate post-ejaculatory 5-fold saline dilution of the ejaculate and/or by washing the spermatozoa twice with BSSA. The washing procedure was started 20 min after ejaculation. Semen samples were obtained from donors A and B with known apparently normal prostatic function. Donor A delivered 8 ejaculates, 4 of which were collected as whole semen, and the remaining four as split ejaculates in 2 portions (I and II) (see "Methods"). Two of the split ejaculates, and two of the whole ejaculates were immediately subjected to BSS dilution. "Prostatic" and "vesicular" fluids (diluted or undiluted) were obtained by centrifugation of portions I and II from all split ejaculates 20 min after ejaculation, and the spermatozoa of portions I were then washed with BSSA. Aliquots of these albumin treated spermatozoa were incubated (60 min) in either BSSA "prostatic fluid" or "vesicular fluid" (Control PF and VF in Fig. 1), and then exposed to SDS. Spermatozoa from the two diluted and the two undiluted whole ejaculates were exposed to SDS 20 min after ejaculation.

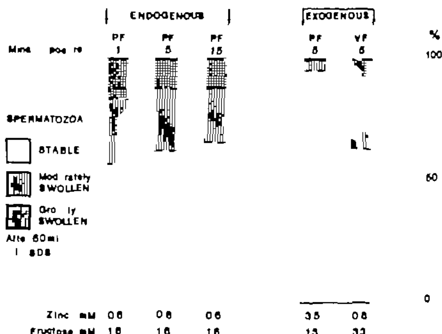


Fig 2 Left Low SDS (sodium dodecyl sulphate) resistance of spermatozoa from a man with impaired prostatic function The spermatozoa were preserved for 1 to 15 min after ejaculation in his own zinc-poor "prostatic fluid" (PF) *Right* Markedly increased SDS resistance induced in an aliquot of the same sperm population after 5 min exposure to exogenous zinc-rich PF from a donor with apparently normal prostatic function. Corresponding exposure to "vesicular fluid" (VF) from this donor had no obvious effect on the spermatozoal SDS resistance

Donor B delivered one whole and one split ejaculate none of them diluted These ejaculates were treated in the same manner as the undiluted ejaculates of donor A with the exception that the incubation period (in Control PF and VF) was reduced to 5 min

The sperm resistance to swelling in SDS under various experimental conditions is shown in Fig. 1 The immediate post-ejaculatory saline dilution of semen from donor A caused a reduction of the percentage SDS resistant spermatozoa Albumin treatment also decreased the SDS resistance and accentuated the effect of dilution (cf thick bars and Control bars respectively) Spermatozoa incubated in PF had a much higher resistance to SDS than their controls. Incubation in VF had not such an effect (Fig 1) This stabilization appeared to be a rapid process since it became fully developed after only 5 min of "prostatic fluid" exposure in donor B

2. *Nature of SDS sensitivity* Each of two donors one with known impaired prostatic function (C) and one with apparently normal prostatic secretion (D) delivered one split ejaculate in two portions (I and II) Aliquots of portion I from both donors were subjected to SDS exposure at 1 5 and 15 min after ejaculation (Fig. 2 left endogenous PF—from donor C) The remaining parts of portions I and portions II were centrifuged to obtain PF and and the sperm pellets of portions I were used continued studies Spermatozoa from donor C were incubated for 5 min in 9 volumes of PF or VF from donor D and then exposed to SDS (Fig 2 right exogenous PF and VF)

The spermatozoa in endogenous (autologous) from donor C showed only a minor increase in the SDS resistance from 1 to 15 min after ejaculation The concentration of zinc and fructose in the were 0 6 and 16 mM respectively

Spermatozoa from the same PF population (donor C) exposed for 5 min to zinc rich PF from donor D became almost maximal SDS resistant Exposure to zinc-poor VF from donor D had such an effect (Fig 2 right PF vs VF)

Spermatozoa from donor D revealed almost maximal SDS resistance already at 1 min after ejaculation This initially high SDS resistance was not reduced by subsequent 5 min exposure zinc-poor PF from donor C The same result was obtained in 5 additional experiments using 5 different donors where the spermatozoa were expo-

15 min to zinc-poor PF In the total material the -an percentage of stable spermatozoa was 93% inge 86–99) before and 94% (84–99) after expo- re to the zinc-poor PF

SCUSSION

e final steps in the male genome transfer occur in ovum Derangement of the sperm head mem- anes is obligatory (Hiramoto 1962) and occurs ncomitant with a rapid decondensation of the clear chromatin (cf Soupart 1976), ultimately aking the male genome available for fusion with female genome To obtain fertilizing ability the ermatozoon has to remain in the female genital ict for some hours but the mechanisms involved *the capacitation process are largely unknown* ustin 1951 Chang 1951 Chang & Hunter 1975) Capacitated spermatozoa have an increased tabol sm and motility in comparison to the un- acitated cells but the motility persists consid bly longer than the fertilizing ability The fertiliz . ability thus seems to be dependent upon other alities than those expressed by the increased otility (cf Chang & Hunter 1975) Capacitation of ermatozoa can also be achieved in vitro by expos- g them to fluids from the female genital tract. The pacitating factor in bovine follicular fluid has een identified as albumin (Lui Cornett & Meizel 77) and albumin is an obligatory component in edia used for in vitro capacitation (Bavister 1969 oupart & Morgenstern 1973) Albumin treated man permatozoa and spermatozoa exposed to man follicular fluid lose about 70–80% of their c content (Johnsen & Eliasson 1976, 1978) rthermore zinc is removed from rat spermatozoa ring their transfer through the female genital tract man & Gould 1958)

Nuclear chromatin decondensation of normal ma- permatozoa in vitro has earlier been reported ly after addition of S-S bond cleaving agents, e.g. TT (cf Bedford 1975) and it has been suggested at the decondensation in vivo is dependent upon S cleaving factors in the ovum (Mahi & Yana- machi 1975) However the results presented re imply that nuclear chromatin decondensa- tion (NCD) can be elicited also by albumin- duced removal of some sperm factor possibly nc Thus, addition of albumin to the wash- g medium conspicuously increased the per ntage spermatozoa undergoing nuclear decon- densation in SDS when the albumin treatment was started 15–20 min after ejaculation. From a physiological point of view it seems of interest that one factor (i.e. albumin) known to be responsible for induction of fertilizing ability of ejaculated spermatozoa in vitro can release a spermatozoal nuclear chromatin decondensation (NCD) ability that is triggered by SDS derangement of membrane structures. Moreover factors in the seminal plas- ma present in the "prostatic fluid" can fully coun- teract this albumin facilitated NCD-ability

Thus the studies performed on experimentally SDS sensitized and natively SDS sensitive sper matozoa provide additional and more obvious evi- dence that a prostatic factor is of major importance for post-ejaculatory inhibition of sperm NCD-abili- ty Facilitated NCD-ability of spermatozoa from normal donors (induced by albumin or revealed by immediate post-ejaculatory dilution of semen) (see Fig. 1) as well as spontaneous NCD-ability of spermatozoa from a donor with impairment of the prostatic function (Fig. 2) could be inhibited by exposing the spermatozoa to normal prostatic fluid In contrast normal vesicular fluid did not have this effect.

Indirect evidence that the prostatic factor acts directly upon spermatozoal structures, and not by inhibiting some seminal plasma chromatin decon- densating system is provided by the observation that stable spermatozoa did not become SDS sen- sitized by 15 min exposure to portion I fluid from a donor with impaired prostatic function (Results C 2).

Bedford Bent & Calvin (1973) reported that se men samples from infertile men frequently con- tained a high percentage of spermatozoa swelling in SDS This was believed to reflect a dysfunction in the spermatogenesis The present and previous (Kvist & Eliasson 1980 Kvist 1980) results dem- onstrate the existence of another possibility i.e impaired function of the prostate Thus sperm NCD-ability not counteracted by a prostatic factor at the time of ejaculation may represent an abnor mality associated with male infertility

The events discussed above provides new con- cepts concerning the physiology of male genome transfer It is suggested that there exists an intrinsic sperm nuclear chromatin decondensation ability which can be triggered by derangement of sperm nuclear membranes, facilitated by albumin and re versibly inhibited by a factor (zinc) in the prostatic

fluid Consequently (albumin induced?) removal of spermatozoal zinc in the female genital tract may be of physiological importance as a prerequisite for the final nuclear chromatin decondensation in the ovum

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[Im]portance of spermatozoal zinc as temporary inhibitor [of] sperm nuclear chromatin decondensation [ab]ility in man

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KVIST U. Importance of spermatozoal zinc as temporary inhibitor of sperm nuclear chromatin decondensation ability in man. Acta Physiol Scand 1980, 109: 79–84. Received 1 Oct. 1979. ISSN 0001-6772. Reproductive Physiology Unit, Department of Physiology, Karolinska Institutet, Stockholm, Sweden.

Nuclear chromatin decondensation (NCD) of ejaculated human spermatozoa was studied in vitro. Spermatozoa subjected to membrane disintegration induced by the detergent sodium dodecyl sulphate (SDS) were found to undergo NCD if previously or afterwards treated with substances known to deplete the spermatozoa of zinc (albumin and EDTA). Zn^{2+} but not other prostatic cations (Ca^{2+}, Mg^{2+}) inhibited the experimentally induced NCD and the NCD of spermatozoa from men with impaired prostatic function. It is suggested that the human spermatozoon has an intrinsic mechanism for NCD that is preserved by temporary zinc inhibition and might be reactivated by zinc removal within the female genital tract.

Key words: Spermatozoa, human, prostate, zinc, EDTA, NCD-ability

[Prev]ious investigations involving determination of [hum]an sperm resistance to nuclear swelling in [sodi]um dodecyl sulphate (SDS) suggest that some [se]minal plasma component of prostatic origin plays [an i]mportant role as a reversible inhibitor of the [sper]m nuclear chromatin decondensatoin (NCD) [abili]ty (Kvist & Eliasson 1980, Kvist 1980a, b). [Di]rect evidence that the crucial factor might be [zinc] was provided by the observation that albumin [know]n to deprive the spermatozoa of zinc) continuously reduced sperm resistance to swelling in [SDS] (Kvist 1980b). Exposure to exogenous Zn^{2+}, treatment with albumin, or another zinc-removing substance (EDTA) were used in the present [stud]y to provide more direct evidence for the im[portance] of zinc as temporary inhibitor of spermat[ozoa]l NCD-ability. Accounts of some of the re[sults] presented here were earlier briefly communi[cate]d (Eliasson & Kvist 1976, Kvist & Eliasson [197]9).

[MA]TERIAL AND METHODS

[Sem]en samples were obtained from men referred to the [labo]ratory for ... barren union, and from volunteers (mainly medical students). All semen samples were collected by masturbation after 3–5 days of continence.

Donors with known impaired prostatic function were chosen to obtain spermatozoa with native sensitivity to react with nuclear swelling in SDS. To obtain semen samples with ... high (normal) sperm SDS-resistance donors with apparently normal prostatic function were selected (Kvist 1980a). Donors with "apparently normal" prostatic function are defined as men who revealed normal values of the biochemical markers for prostatic secretion in seminal plasma (Zn >1.2 mM, Mg >2.9 mM, acid phosphatase activity >6.9 mkat/l) as well as for seminal vesicular secretion (fructose >6.7 mM). Donors with impaired prostatic secretion had revealed subnormal seminal plasma concentrations of the prostatic components (cf. Eliasson 1975). Zinc and magnesium concentration in seminal plasma were measured with an atomic absorption spectrophotometer (cf. Eliasson & Lindholmer 1971, 1972) and the acid phosphatase activity according to Sigma Technical Bulletin No 104. Fructose concentration was determined colorimetrically (Karvonen & Malm 1955).

Unwashed spermatozoa were studied in aliquots of whole semen. Washed spermatozoa were prepared by centrifugation of semen (400 g, 15 min) followed by removal of the supernatant. The sperm pellets were thereafter resuspended in ... buffered salt solution (BSS) and recentrifugated. The washing procedure was then repeated once. The composition of BSS was (mM) NaCl 123, KCl 5, HCl ≈4, TRIS 37, pH 8.0. An alternative

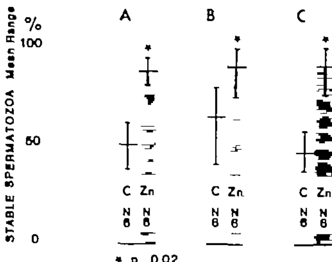


Fig 1 Zinc-induced normalization of sperm resistance to nuclear swelling in sodium dodecyl sulphate (SDS) of natively SDS sensitive spermatozoa from men with impaired prostatic function The spermatozoa were exposed to SDS min after zinc treatment. (A) Semen mixed with the equal amount of BSS (*C*) or BSS containing 3 mM Zn^{2+} (*Zn*) 2 min after ejaculation (B) The same proceedings as in (A) performed 90 min after ejaculation (C) SDS resistance of washed spermatozoa without (*C*) or with the addition of Zn^{2+} (1 6 mM) to the suspension medium

washing method used to obtain SDS sensitized spermatozoa (Kvist 1980*b*) was washing in BSS containing 4% albumin (BSSA) When the influence of divalent metallic cations normally present in prostatic fluid (zinc calcium or magnesium) were studied water solutions of ZnSO CaCl were added to BSS in a volume relation of 1 100 In experiments involving sperm treatment with disodium-aethylen-diamin-tetraacetate (EDTA) a water solution of EDTA was added to BSS or SDS solution in a volume relation of 3 100 providing a final EDTA concentration of 6 mM

The degree of nuclear chromatin resistance to undergo NCD was assessed as the degree of nuclear swelling after the spermatozoa had been exposed for 60 minutes (when not indicated otherwise) to 1% SDS in 0 05 M borate buffer pH 9 0 One volume of semen or sperm suspension was mixed with 9 volumes of SDS solution and the reaction was stopped by adding the equal amount of 5% glutaraldehyde in 0.05 borate buffer pH 9 0 In 1 expt where the spermatozoa had been pre-exposed to SDS the NCD was assessed after 60 min incubation in BSS or in BSS containing 6 mM EDTA

The degree of nuclear swelling was assessed with phase contrast microscopy (500×) and the spermatozoa were classified as stable (=not swollen) and unstable (=moderately and grossly swollen) (Kvist & Eliasson 1980)

For descriptive statistics mean and range is given for determined percentage of stable spermatozoa and analytical statistics was performed with the Randomization Test for Matched Pairs and the Mann-Whitney U-test for independent groups (cf Siegel 1956)

RESULTS

Effect of zinc on natively SDS sensitive spermatozoa

Donors ($N=1?$) with known impaired pr function were selected to obtain semen sa containing spermatozoa with natively low re ance to nuclear swelling in SDS The effect of e genous zinc on the SDS resistance of the spe tozoa was studied under three different cond (series A B and C)

In series A (6 samples 4 donors) an aliquo each semen sample was gently mixed with the e amount of BSS containing zinc (3 ? mM) at after ejaculation Aliquots mixed with the amount of zinc-free BSS served as controls 2 min thereafter all specimens were subjecte SDS treatment As shown in Fig 1 A zinc trea conspicuously increased the percentage stable swelling) spermatozoa (from mean of 51 to $p<0\ 02$)

The experimental proceeding in series B (6 ples 6 donors) was the same as in serie A wi exception that the addition of zinc-contain zinc free BSS was not made until approximatel minutes after ejaculation In this case the mean centage of stable spermatozoa in the control spe mens was somewhat higher than in series A H ever also here zinc addition induced a signif increase in the percentage stable spermatozoa a mean of 65 to 91 $p<0\ 0?$) (Fig. 1B)

The material in series C (6 samples 4 do consisted of spermatozoa washed *in BSS* app mately 90 min after ejaculation Aliquots of sperm population were then resuspended in BSS (controls) or in BSS containing zinc (1 6 and were then within ? min exposed to SDS shown in Fig 1C zinc treatment of the wa specimens caused an increase in percentage st spermatozoa ($p<0\ 0?$) from a mean of 47 to 91

Effect of zinc on experimentally increased sperm SDS sensitivity

(1) *Albumin enhanced sensitivity* Semen sa ($N=5$) from 5 donors with impaired prostatic f tion were subjected to BSSA washing 15 min a ejaculation in order to study the effect of zi sperm population supersensitized to SDS (K 1980*b*) After BSSA washing sperm aliquot each sample were resuspended in pure BSS or BSS containing zinc (1 6 mM) and the sperm

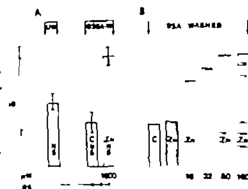


Fig. 2. Effect of zinc on albumin-enhanced sperm nuclear chromatin decondensation in SDS. (A) Spermatozoa from ... with impaired prostatic function. UW = Unwashed spermatozoa. BSSA-W = Spermatozoa washed in buffer solution containing albumin. C = Control. Zn = Zinc exposed spermatozoa. (B) Concentration dependent effect of zinc on the sperm resistance to SDS of BSSA-washed spermatozoa.

... than 2 min subjected to SDS treatment. For comparison an aliquot of each semen sample (i.e. ...shed, not albumin treated) was subjected to SDS treatment at 15 min after ejaculation.

BSSA-washing increased the percentage of spermatozoa swelling in SDS (Fig. 2A (C) vs. (UW) $p<0.05$). Addition of zinc to the BSSA-washed specimens not only abolished the albumin induced effect, but even induced an SDS-resistance considerably higher than that observed in the unwashed specimens ((Zn) vs. (C) and vs. (UW) in Fig ... $p<0.05$).

In another experiment pooled BSSA-washed spermatozoa were used to study the effect of different concentrations of zinc on the sperm resistance to SDS. Sperm aliquots were resuspended in ... BSS or BSS containing gradually increasing concentration of zinc and were then within ... min exposed to SDS. As shown in Fig. B, zinc concentration of 80 μM in the medium was sufficient to increase the proportion stable spermatozoa to over ...

EDTA induced sensitivity. Semen samples ... from different donors with apparently normal prostatic function were used. Within ... h after ejaculation each semen sample was divided in 3 fractions (I, II, III). In order to determine the pre...ting sperm SDS resistance fractions I were washed twice in BSS, resuspended in BSS and then immediately subjected to SDS treatment. Fractions II and III were first washed once in BSS and then in BSS containing EDTA (6 mM). Before SDS treatment fractions II were resuspended in pure BSS whereas fractions III were resuspended in BSS containing zinc (0.16 mM). As shown in Fig. 3A the mean percentage SDS resistant spermatozoa in fractions I was 84. EDTA treatment reduced the percentage stable spermatozoa to 28 (II vs. I in Fig. 3A $p<0.05$). However, the addition of zinc to previously EDTA-exposed specimens induced almost full (98%) sperm resistance to SDS (III vs. II and vs. I in Fig. 3A, both differences $p<0.05$).

The observation that EDTA pre-treatment conspicuously increased sperm sensitivity to subsequent SDS exposure made it interesting to study whether EDTA per se would induce nuclear chromatin decondensation of spermatozoa which previously had been briefly exposed to SDS. Randomly selected semen samples ($N=4$) were pooled and divided in two groups. Both groups were centrifuged and the spermatozoa were resuspended in SDS solution containing zinc (0.16 mM) and thereafter immediately centrifuged (total time of SDS exposure 10 min). After an intermediate BSS wash

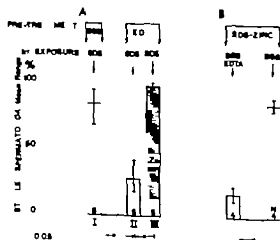


Fig. 3. (A) Effect of zinc on EDTA induced sperm NCD in SDS. I = Spermatozoa washed twice in buffered salt solution (BSS) before SDS exposure. II = BSS-EDTA washed spermatozoa. III = BSS-EDTA washed spermatozoa exposed to Zn^{2+} (0.16 mM) ... min before SDS treatment. (B) SDS-Zinc pre-treated spermatozoa incubated for 1 h in BSS-EDTA (left) and in pure BSS (right). Note that the presence of zinc (0.16 mM) in the SDS-solution did not interact with the SDS dependent part of the NCD process and that the presence of SDS is not needed during the second EDTA-induced part of this process.

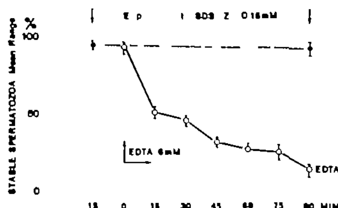


Fig 4 Dynamics of EDTA-induced NCD studied in 7 groups of washed spermatozoa in SDS containing 0 16 mM Zn (*open circles*) For comparison is shown the inability of SDS as such to induce NCD (*closed circles*)

the spermatozoa of one group were resuspended in BSS and those of the other group in BSS containing EDTA (6 mM) Both groups were divided in 4 fractions and stored for one hour before assessment of the degree of nuclear chromatin swelling As shown in Fig 3 B a high proportion of spermatozoa pre-treated with SDS-Zn remained stable (mean 84% stable spermatozoa) when subsequently incubated in pure BSS whereas most of similarly pre-treated spermatozoa underwent nuclear decondensation when incubated in BSS containing EDTA (mean 27% stable spermatozoa, differences from pure BSS-incubated $p<0.05$)

Zinc treated spermatozoa were used to study the time dependence of EDTA induced sperm swelling in SDS The material consisted of randomly selected semen samples ($N=5$) which were pooled and washed in BSS containing zinc (0 16 mM) The spermatozoa were resuspended in SDS solution containing zinc (0 16 mM) The suspension was immediately divided in 9 groups each consisting 4 fractions To seven of the groups EDTA was added (final concentration 6 mM) after 15 min of SDS exposure and the nuclear swelling reaction was studied in these groups at 15 min intervals from time 0 to 90 min (open circles in Fig 4) The remaining two groups did not receive EDTA Here the percentage stable spermatozoa was assessed immediately after SDS exposure and 105 min later (closed circles in Fig 4) As shown in Fig 4 addition of EDTA to the SDS-Zn sperm suspensions reduced the percentage stable spermatozoa with time whereas simply SDS-Zn exposure for 105 min did not reduce the percentage stable spermatozoa significantly

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The concept of a sperm NCD ability preserved by zinc appears compatible with the facts that (*a*) the spermatozoon has to remain in the female genital tract for several hours in order to gain fertilizing ability (cf. Chang & Hunter 1975) and it releases zinc during this period (Gunn & Gould 1958), (*b*) the fertilizing ability is again lost upon sperm re-exposure to seminal plasma making a renewed incubation in the female genital tract necessary to regain fertilizing ability (Chang 1957), (*c*) fertilizing ability can be induced in vitro by sperm exposure to media where albumin seems to be an obligate constituent (Soupart & Morgenstern 1973) and (*d*) in vivo NCD starts concomitant with sperm membrane disintegration, i.e. *before* the entire sperm head is engulfed by the ooplasm (Barros & Franklin 1968, Bedford 1972, Soupart 1976).

In conclusion, the present and previous studies on human spermatozoa (Kvist & Eliasson 1980, Kvist 1980*a*, *b*...) support the idea that the sper...

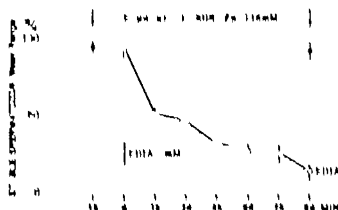


Fig. 4. Dynamics of EDTA-induced NCD studied in 2 groups of washed spermatozoa in SDS containing 0.16 mM Zn (open circles). For comparison is shown the instability of SDS as such [illegible] (*Fig. 1*, [illegible]).

the spermatozoa of one group were resuspended in BSS and those of the other group in BSS containing EDTA (6 mM). Both groups were divided into 4 fractions and stored for one h before assessment of the degree of nuclear chromatin swelling. As shown in Fig. 3B a high proportion of spermatozoa pre-treated with SDS-Zn remained stable (mean 84% stable spermatozoa) when subsequently incubated in pure BSS, whereas most of similarly pre-treated spermatozoa underwent decondensation when incubated in BSS containing EDTA (mean 27% stable spermatozoa; difference from pure BSS incubate $p<0.05$).

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Other prostatic divalent cations and sperm SDS sensitivity

Spermatozoa (BSSA washed 2 h after ejaculation) were used to study whether in addition to zinc, other divalent cations present in the prostatic [illegible] (magnesium and calcium) would counteract experimentally induced sperm SDS sensitivity. The material consisted of semen samples (N=9) from fertile donors with apparently normal [illegible] function. Each BSSA washed sample was divided in 4 fractions. One fraction served as control, the other 3 fractions with either Mg^{2+}, Ca^{2+} or Zn^{2+} added (final concentrations 1.6 mM). Thereafter all fractions were subjected to SDS treatment. The presence of Mg^{2+} and Ca^{2+} did not change the percentage of SDS-resistant spermatozoa from the initial level (mean and range: Mg^{2+} [illegible]; Ca^{2+} [illegible] (34–78) and controls [illegible]) whereas the presence of Zn^{2+} in the same concentration induced almost complete sperm SDS resistance (mean and range [illegible] (89–97)) (Zn^{2+} treated vs Mg^{2+}, Ca^{2+} and controls $p<0.05$).

DISCUSSION

For normal [illegible] it is essential that the sperm nuclear chromatin decondenses at the appropriate moment, i.e. when the spermatozoon penetrates into the ovum (Bedford [illegible] 1968; cf. Sanger [illegible] 1976). If for some reason the chromatin remains condensed after penetration, fertilization is [illegible]. [illegible] Nuclear decondensation presumably is [illegible] requisite [illegible] sperm nuclear disintegration of sperm head membranes ([illegible] 1962) and cleavage of S-S bridges between chromatin protein subunits (cf. [illegible] 1978). During the process of penetration the head membranes disintegrate (cf. Sanger [illegible]) and it has been postulated that the ovum provides the [illegible] necessary to reduce the [illegible] S-S bridges in the sperm chromatin (Calvin & [illegible] 1971; Mahi & Yanagimachi 1975). The formation of chromatin-stabilizing S-S bonds is evidently accomplished already during sperm passage through the epididymis (Calvin & Bedford [illegible]; Calvin & [illegible] 1975). Recent studies on human spermatozoa (Kvist & Eliasson 1980[illegible]; 1980*a*, *b*) have revealed that a factor of prostatic origin efficiently contributes to maintain the [illegible] of condensed sperm nuclear chromatin [illegible] semen from men with impaired prostatic function.

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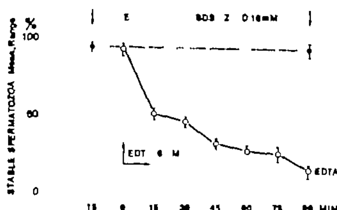


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Physiol Scand 1980, 109: 85–88

[illegible]covery of choline acetyltransferase activity [illegible] the rat urinary bladder deprived of half [illegible] its innervation

[illegible]THER BANNS, JÖRGEN EKSTRÖM and STEPHEN P. MANN
[illegible]rtment of Physiology and Biophysics, University of Lund, Sweden and Agricul[illegible] Research Council Institute of Animal Physiology, Babraham, Cambridge, England

BANNS, H., EKSTRÖM, J. & MANN, S. P.: Recovery of choline acetyltransferase activity in the rat urinary bladder deprived of half of its innervation. Acta Physiol Scand 1980, 109: 85–88. Received 4 Sept. 1979. ISSN 0001-6772. Department of Physiology and Biophysics, University of Lund, Sweden and Agricultural Research Council Institute of Animal Physiology, Barbraham, Cambridge, England.

In the urinary bladder of the rat, partially denervated by unilateral removal of the pelvic ganglion 3 days in advance, the activity of the acetylcholine-forming enzyme, choline acetyltransferase, measured by radiolabelling method, was reduced to 58 % of the control. A gain in enzyme activity of 28 % was found to have occurred when the bladders were examined 25 days postoperatively: the main part of this increase took place during the period 3 to 6 days after the operation and beyond 25 days no further gain in enzyme activity was found. The present findings are compared with previous observations of transient supersensitivity and an increased motor response to electrical stimulation of the intact pelvic nerve of such partially denervated bladder.

Key words: Choline acetyltransferase activity, denervation, nerve sprouting, urinary bladder, rat

[illegible] urinary bladder of the rat receives its cholinergic innervation both from the pelvic and the [illegible]ogastric nerve (see Elmér 1978). On their way to [illegible] bladder most hypogastric fibres pass the pelvic [illegible]lion (Langworthy 1965, Purinton et al. 1973). [illegible]ity of the acetylcholine synthesizing enzyme [illegible]ne acetyltransferase, has been demonstrated in rat urinary bladder (Ekström 1975), and bilateral [illegible]oval of the pelvic ganglion reduces the activity [illegible]his enzyme to 5 % or less of the normal value [illegible]ström & Elmér 1977). If, on the other hand, the [illegible]ogastric nerve is cut bilaterally only a small [illegible]action of about 16 % of short duration, is ob[illegible]ed, being obvious on the 3rd but not on the 8th [illegible] postoperatively; on the 8th day the enzyme [illegible]ity tended, in fact, to be higher than normal [illegible]ström & Elmér 1977).

[illegible]he present study was undertaken to investigate [illegible]ether a rapid restitution in activity of choline [illegible]tyltransferase from a low level would occur after [illegible]lateral removal of the pelvic ganglion. The urinary bladders were examined 3, 6, 25 and 60 days postoperatively.

A preliminary account of some of the findings has been given (Banns et al. 1978).

MATERIAL AND METHODS

65 male adult rats of a Sprague-Dawley strain bred at the department of Physiology, Lund, were used. At the end of the experimental period they weighed 342±4 g (mean±S.E.). Under ether anesthesia the abdomen was opened and with the aid of a dissecting microscope, the left pelvic ganglion with its nerve filaments close to the bladder was removed. The wound was then sutured. The animals were killed by inhalation of ether. The bladders were removed, cleaned and weighed; they were then stored at −20°C.

At each time of observation 8 operated rats and their litter mate controls were examined except for the observation at 60 days when the number of operated rats was seven.

In 3 rats both the left and the right pelvic ganglion and their nerve filaments were removed. The bladders of these rats had to be emptied daily by manual pressure under

matozoon has an intrinsic mechanism for NCD that is preserved by temporary zinc inhibition and might be reactivated by zinc removal within the female genital tract

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der was attempted, the mean bladder weight 178.7±10.8 mg 3 days postoperatively. In one [th]ese bladders, the acetylcholine-forming capaci[ty w]as below the limit of detection. In the others it [was] 4 and 1.7 nmol acetylcholine per h, respec[tive]ly.

[DIS]CUSSION

[C]holine acetyltransferase studied in the present [inve]stigation by a radioactive method was probably [of n]euronal origin, since a profound and rapid fall in [its] activity occurred following bilateral removal of [the] pelvic ganglion; the same result has previously [been] obtained by the use of a bioassay method [(Ek]ström & Elmér 1977).

[In] the urinary bladder, partially denervated by [unil]ateral removal of the pelvic ganglion, the activi[ty of] choline acetyltransferase was found to be 58% [of th]e control, 3 days postoperatively. An increase [of] 15% from this level of enzyme activity was [demo]nstrated. It was particularly obvious at an ear[ly st]age, between 3 and 6 days, and was found to [have] come to an end already when the bladders [were] examined 25 days after the operation. Similar[ly, th]e restitution of enzyme activity in the urinary [blad]ders after cutting the hypogastric nerve bilater[ally] occurred at an early stage (Ekström & Elmér [197]7).

[It i]s of interest to compare the findings of the [pres]ent investigation with those of Ekström, Elmér [& B]anns (1978) showing that the supersensitivity [deve]loped in the urinary bladder to the parasym[path]omimetic substance methacholine after remov[al o]f the pelvic ganglion on one side was only of a [tran]sient nature, being obvious 6–7 days but not [30] days after the operation, and further that the [resp]onse of the bladder to electrical stimulation of [the i]ntact pelvic nerve was found to be increased at [the] later observation in spite of the disappearance [of th]e sensitization (Ekström & Elmér unpublished [obs]ervation). The activity of choline acetyl[tra]nsferase has been shown to be dependent on the [traf]fic of nerve impulses (see Ekström 1978). The [reco]very in choline acetyltransferase activity re[por]ted in the present study may be a consequence [of a] compensatory increase in the traffic of motor [imp]ulses in the remaining nervous pathway. This i[s, ho]wever probably only part of the explanation; the [disa]ppearance of the supersensitivity to metha-choline and the increased motor responses elicited by nerve stimulation suggest outgrowth of nerves from the intact nervous pathway; this may also be suggested by the finding that the weight of the partially denervated bladders seemed to fall at the later stages of the period of observation, although still being higher than the controls. In the previous work by Ekström, Elmér & Banns (1978) there was no longer any significant difference between the weight of the partially denervated bladder and the control 25–30 days after the operation. A favourable condition for nerve sprouting seems to exist when degenerating and intact nerves intermingle with each other as judged from morphological and functional studies on partially denervated skeletal muscles (see Edds 1953) and sympathetic ganglia (Murray & Thompson 1957). In the urinary bladder, such a favourable condition may also occur since the pelvic nerve of each side distributes bilaterally (Carpenter & Rubin 1967, Elmér 1975).

This work was supported by travelling grants to J. E. from the Swedish Medical Research Council (K77-60F 5081-01, K77-14R 5081) and from Lilla Kungliga Läkaresällskapet i Lund, and also by grants from Harald och Greta Jeanssons stiftelser.

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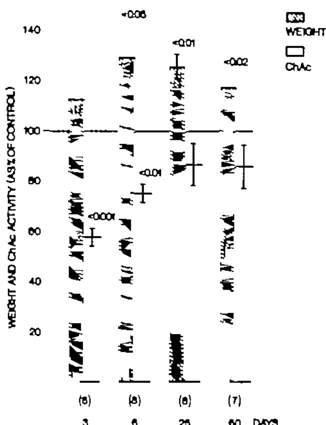


Fig 1 Weight and choline acetyltransferase activity of urinary bladders at different time intervals after unilateral removal of the pelvic ganglion. The weight and the enzyme activity are expressed as a percentage of those of the urinary bladders of the control litter mate (mean± S E) Number of comparisons are shown in brackets and when the differences are significant the *P*-values are given.

ether anesthesia To avoid bacterial infections 70 mg of a sulfonamide (Sulfuno® Nordmark Werke) was given s c each day The animals were killed 3 days postoperatively

Preparation of the urinary bladder The bladders were homogenized in 1 ml Na-phosphate buffer 0 1 M pH 6.5 containing dithiothreitol 1.2 mM using a Polytron homogenizer at high speed for 15 s The homogenate was then usually stored at −20°C for a few days before being analysed

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The medium used for test and control incubation is given in Banns Ekström & Mann (1979) the control incubation contained acetylcholinesterase instead of eserine Assays both test and control were carried out in duplicate each tube containing 85 µl of the incubation medium and 25 µl of tissue homogenate The reaction was started by addition of 10 µl [methyl- H] acetyl-CoA and after 30 min at 39°C it was stopped by addition of 80 µl of chloric acid followed by cooling in an icebath

Using columns of DeAcidite FFIP resin in the ch form [methyl-³H] acetyl-CoA was separated from a choline (see Hebb et al 1975) The true reading for choline formation was obtained by subtracting the r of radioactivity left in the control incubations acetylcholine was continually destroyed from the tained in the test incubations where acetylcholine preserved (see Banns et al 1979)

The enzyme activity is expressed in nmol acetylch produced/bladder h (total activity) the figures corrected for the volume of the bladders

Statistics Student s *t*-test was used paired comparisons were made between the operated animal and control litter mate *P*-values of less than 0 05 were considered significant.

RESULTS

Neither at the start nor at the end of the experiments was there any difference in body-w between the operated rats and their controls.

The mean weight and the mean choline acetyltransferase activity of the urinary bladders of whole group of 31 control animals was 72.2±1 1 and 38 7±1 8 nmol acetylcholine formed per bladder per h respectively

The weight of the partially denervated bladders was significantly higher than their controls except for the earliest observation where the difference only tended to be significant (0 05<*P*<0.1). When expressed as a percentage the denervated bladders weighed 113 (3 days) 130 (6 days) 126 (25 days) 118 (60 days) % of their respective controls (see Fig 1)

The activity of choline acetyltransferase in partially denervated urinary bladders was significantly lowered 3 and 6 days postoperatively it was 58 and 75% of their corresponding control bladders respectively (see Fig 1) There was no significant difference between denervated and control bladders 25 and 60 days postoperatively though the mean percentage figures obtained at these stages were still below the 100% score both occasions they were 86

The choline acetyltransferase activity obtained days postoperatively in the partially denervated bladders expressed as a percentage differed significantly from those obtained 6 (*P*<0 05) (*P*<0 01) and 60 (*P*<0 01) days after operation

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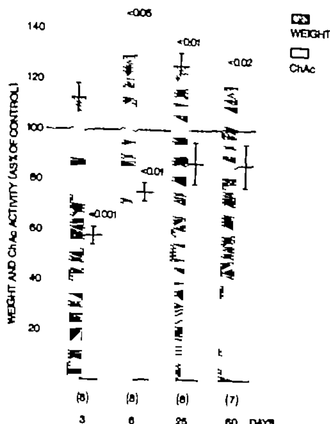


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Physiol Scand 1980 109 89–95

...nctional and metabolic effects of terbutaline ...d propranolol in fast and slow contracting ...eletal muscle in vitro

K FELLENIUS ROLAND HEDBERG EVA HOLMBERG and BERTIL WALDECK

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FELLENIUS E., HEDBERG R., HOLMBERG E. & WALDECK, B., Functional and metabolic effects of terbutaline and propranolol in fast and slow-contracting skeletal muscle in vitro Acta Physiol Scand 1980, 109 89–95 Received 6 Sept. 1979 ISSN 0001-6772. Research and Development Laboratories, AB Hässle Mölndal, Department of Zoophysiology University of Uppsala and Research and Development Laboratories, AB Draco Lund, S eden.

The soleus, a slow-contracting, and the extensor digitorum longus (EDL), fast-contracting skeletal muscle from guinea-pig were prepared for isometric recording of sub-tetanic contractions in vitro. The content of adenosine-triphosphate (ATP) and creatinephosphate (CP) together ith their metabolites and the contents of lactate, pyruvate and cyclic adenosine mo ophosphate (AMP) in the muscles were determined. It was found that the energy and redox state of the isolated soleus and EDL muscles is very stable and does not significantly differ from the normal state in vivo. Moreover there were no consistent changes in these variables after treatment with terbutaline (a β_2-adrenoceptor agonist) or propranolol or both. Thus, effects on energy metabolism do not seem to cause the changes in muscle contraction, characteristic for β-adrenoceptor stimulation. On the other hand, the functional effects were accompanied by elevation of the c AMP level of the muscles.

Key words: β-adrenoceptor terbutaline propranolol, skeletal muscle contraction energy state redox state guinea-pig

...rbutaline and related compounds have profound ...cts on the contraction pattern of skeletal mus... In slow-contracting muscles the tension and ...sion of maximal twitches is decreased, resulting ... decrease in the tension and degree of fusion of ...tetanic contractions. Fast-contracting muscles ...pond with an increase in these variables (Bow...n & Zaimis 1958 Bowman & Nott 1969). These ...cts on skeletal muscle contraction may have ...ical implications Firstly during treatment with ...adrenoceptor agonist, muscle tremor appears as ...ose-limiting side-effect (e.g. Thiringer & Sved... 1975 Brittain & Jack 1976). This effect may be ...to a reduced degree of fusion in slow-contract... muscles (Bowman & Nott 1969) Secondly ...atment with β-adrenoceptor antagonists often ...ses a sensation of muscle fatigue (Banks et al ...8, Fellenius et al. 1979) Among several factors ...t may be the direct cause of muscle weakness, the importance of cell energy storage compounds has been stressed (Edwards 1978) Lactate accumulation or associated pH changes due to redox changes may be additional factors responsible for the impaired muscle function (Tesch et al 1978)

Isolated preparations of the slow-contracting soleus and the fast-contracting extensor digitorum longus (EDL) muscles from the guinea-pig have proved useful in the study of the sympathomimetic-induced effects on skeletal muscle contractions (Tashiro 1973 Waldeck 1977 Holmberg & Waldeck 1977 Al Jeboory & Marshall 1978). Thus the previous in vivo observation that the effects are mediated via β_2-adrenoceptors (Bowman & Nott 1970 Bohmer & Raper 1976) has been convincingly confirmed. In the present study we have tried to investigate whether the functional effects of terbutaline a

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...ctional and metabolic effects of terbutaline ...l propranolol in fast and slow-contracting ...letal muscle in vitro

... FELLENIUS, ROLAND HEDBERG EVA HOLMBERG and BERTIL WALDECK

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FELLENIUS E., HEDBERG, R., HOLMBERG, E. & WALDECK, B Functional and metabolic effects of terbutaline and propranolol in fast and slow-contracting skeletal muscle in vitro. Acta Physiol Scand 1980 109 89-95 Received 6 Sept. 1979 ISSN 0001-6772. Research and Development Laboratories AB Hässle Mölndal, Department of Zoophysiology University of Uppsala and Research and Development Laboratories AB Draco Lund, Sweden.

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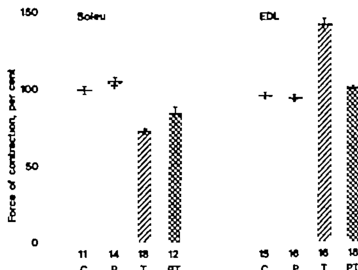


Fig 1 Effects of propranolol and terbutaline on subtetanic isometric contractions of isolated soleus and EDL muscles Propranolol (P 0 03 μmol/l) was added to the baths, followed 10 min later by terbutaline (T 0.3 μmol/l) The experiment was stopped after another 20 min Muscles treated with either drug alone and untreated muscles (C) were run in parallel The result is expressed in per cent of the force developed by each individual muscle 30 min before the termination of the expt Means ± S E. and at the bottom of each bar the number of expts.

selective β_2-adrenoceptor agonist (Bergman et al 1969) are accompanied by changes in energy and redox state In some experiments propranolol was used to block the effects of terbutaline The possible role of c AMP as mediator of functional and metabolic effects was also studied The energy charge and redox state in the isolated in vitro preparation was compared with the corresponding figure in vivo in order to evaluate the viability of the in-vitro preparation

METHODS

(*a*) *Animal treatment* Male guinea-pigs weighing about 200 g were used The soleus and the EDL muscles were dissected out under pentobarbitone anesthesia and mounted in organ baths containing oxygenated Krebs solution at 37° Subtetanic contractions were evoked by transmural field stimulation every 15 s throughout the experiment (Waldeck 1976 Holmberg & Waldeck 1977)

The muscle preparations were allowed to stabilize for 1 h after which propranolol (0 03 μmol/l) was added followed 10 min later by terbutalme (0 3 μmol/l). After another 20 min the stimulation was stopped and the muscles were quickly removed wiped off and dropped into liquid nitrogen. Muscles treated with either propranolol or terbutalme and untreated muscles (control) were run in parallel In some control expts the stimulation was stopped 30 min before the end of the expt. (resting muscles) In order to make comparisons with the metabolic state under in-vivo conditions some muscles were dropped into liquid nitrogen immediately after they had been dissected out The average weight ± S D was 86± 20 mg for the soleus and 124±22 mg for the EDL muscles were pooled 3 by 3 and stored in liquid [illegible] or packed in dry ice until the biochemical analysis

(*b*) *Analytical method* The frozen tissue homogenized and treated with 0 6 mol/l HClO [illegible] previously described (Karlsson et al 1975) The perchloric extract obtained was analyzed for adenosine-triphosphate (ATP) (Lamprecht & Trautschold 1962), adenosine diphosphate (ADP) and adenosine-monophosphate (A[illegible] (Adam 196[illegible]) creatinephosphate (CP) (Lamprecht Stein 196[illegible]) creatine (C) (Bernt et al 1962) and lactate pyruvate (Hohorst et al 1959) The content of c AMP analyzed by means of a protein-binding assay (The clic AMP Assay The Radiochemical Centre Amersham England Jan 1975)

(*c*) *Statistical analysis* Statistical analysis was formed using analysis of variance followed by the [illegible] Values of $P<0\ 05$ were considered statistically significant

RESULTS

(*a*) *Functional effects*

In the soleus muscle terbutaline (0 3 μmol/l) depressed the subtetanic contractions by about [illegible] ($P<0\ 001$) (Fig 1) Propranolol (0 03 μmol/l) had no significant effect per se but attenuated the effect of terbutaline ($P<0\ 005$) In the EDL the force of subtetanic contractions increased by about 40 % after the addition of terbutaline ($P<0\ 001$) This effect was completely blocked by propranolol ($P<0$[illegible] which by itself was devoid of effect compared to the control expt

1 The con entration of compounds involved i energy metabolism of the resting and working soleus DL muscles nd th effect of propranolol (0.03 μmol/l) and terbutaline (0.3 μmol/l)

± S.E. in mmol/kg et weight and the number of determinations, each based on three pooled muscles. For r details, see legend to Fig. 1 and the methods section

le and le	Resting muscles		Working muscles, in vitro			
	I vivo	In vitro	Control	Propranolol	Terbutaline	Propr + Terb
	3.2 ±0.3 (5)	2.8 ±0.2 (4)	2.5 ±0.3 (4)	2.5 ±0.1 (4)	3 1 ±0.2 (4)	2.9 ±0.2 (5)
	0 73±0.04 (5)	0.68±0 03 (4)	0 63±0.09 (4)	0.55±0 09 (4)	0 76 0 05 (4)	0 67±0 07 (5)
	0 07±0 01 (5)	0 03±0 01 (4)	0.06±0 01 (4)	0 02±0.01 (4)	0 03±0.01 (4)	0.03±0.01 (5)
	7 2 ±0 6 (5)	8 7 ±0 6 (4)	7.2 ±1.0 (4)	6.2 ±0.. (4)	7 6 ±0 7 (4)	6.5 ±1 0 (5)
	6 2 +0 6 (5)	4 ±0.8 (4)	6.9 ±0.7 (4)	5.3 ±1.6 (4)	5.8 ±0.8 (4)	4 7 ±0 4 (5)
	4 7 ±0.3 (5)	3.3 ±0.. (4)	3 9 ±0.3 (5)	4.0 ±0.1 (5)	3.6 ±0.2 (4)	3 8 ±0.3 (5)
	0.75±0 01 (5)	0.63±0.04 (4)	0.59±0.03 (5)	0 66±0 03 (5)	0.63±0.03 (4)	0.55±0 02 (5)
	0 05±0.01 (5)	0 03±0 01 (4)	0.03±0.01 (5)	0.03±0.01 (5)	0 07±0.00 (4)	0 02±0.01 (5)
	11 6 ±0 6 (5)	14 1 ±0 8 (3)	14 1 ±0.4 (5)	1..8 ±0.8 (5)	11 1 ±0.6 (4)	12.9 ±0 8 (5)
	14 5 ±1 1 (5)	7.3 ±1 1 (4)	10.9 ±1 7 (5)	9 7 ±1.0 (5)	10.4 ±0 7 (4)	11.3 ±2.5 (5)

ffect on the energy st t in resting orking ontrol muscle

results from the determination of ATP and CP ther with their metabolites are shown in Table comparison between resting muscles showed the level of AMP in the soleus muscle in vitro half of that in vivo ($P<0.005$). The same tend- was seen in the EDL muscle ($P<0.05$). The level was about 40% lower in vitro than in vivo ($P<0.05$). The level of C in EDL in vitro was half that found in vivo ($P<0.001$) and a similar direction of effect was also seen in the soleus muscle ($P<0.05$). The product [ATP] [AMP] [ADP]⁻ and [ATP] [C] [ADP]⁻¹ [CP]⁻ reflecting the equilibrium state of the adenylate kinase and creatine phosphokinase reaction, respectively tended to be slightly displaced in the direction of ATP-break down in vitro compared with in vivo (Table ?) but

le 2 The energy state in the resting and orking soleus and EDL muscles and the effect of propranolol) μmol/l) and terbutaline (0.3 μmol/l)

ratios ± S.E. and the number of determinations, each based on three pooled muscles. For further details, see d to Fig. 1 and the methods section

le and ble	Resting muscles		Working muscles, in vitro			
	In vivo	In vitro	Control	Propranolol	Terbutaline	Propr + Terb.
P] [AMP] / DP]	0.4 ±0 04 (5)	0.2 ±0 07 (4)	0.4 ±0.05 (4)	0.3 ±0 05 (4)	0.2 ±0.05 (4)	0.2 ±0 03 (5)
P] [C] / P] [CP]	3.8 ±0.3 (5)	1.2 ±0.4 (4)	4 0 ±0.3 (4)	5.0 ±2.5 (4)	3.2 ±0 6 (4)	3.5 ±0.5 (5)
	0.89+0 01 (5)	0.88±0 01 (4)	0.87±0.01 (4)	0.89±0 01 (4)	0.88±0.00 (4)	0 89±0 00 (5)
P] [AMP] / ADP]	0.4 ±0 04 (5)	0 2 +0 05 (4)	0 3 ±0 11 (5)	0.3 ±0 06 (5)	0.2 ±0.04 (4)	0.4 ±0.21 (5)
TP] [C] / P] [CP]	8.1 ±1 2 (5)	6 6 ±3 6 (4)	5.4 ±1.5 (5)	4.5 ±0 4 (5)	5.3 ±0.3 (4)	6.0 ±2.0 (5)
	0 91±0 00 (5)	0.90±0 01 (4)	0 91±0 01 (5)	0 91±0 00 (5)	0.91±0 01 4)	0.92±0.00 (5)

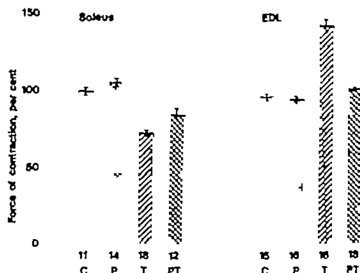


Fig 1 Effects of propranolol and terbutaline on subtetanic isometric contractions of isolated soleus and EDL muscles. Propranolol (P 0 03 μmol/l) was added to the baths, followed 10 min later by terbutaline (T 0 3 μmol/l) The experiment was stopped after another 30 min. Muscles treated with either drug alone and untreated muscles (C) were run in parallel The result is expressed in per cent of the force developed by each individual muscle 30 min before the termination of the expt Means ± S.E and at the bottom of each bar the number of expts

selective β_2-adrenoceptor agonist (Bergman et al 1969) are accompanied by changes in energy and redox state In some experiments propranolol was used to block the effects of terbutaline The possible role of c AMP as mediator of functional and metabolic effects was also studied The energy charge and redox state in the isolated in vitro preparation was compared with the corresponding figure in vivo in order to evaluate the viability of the in vitro preparation

METHODS

(a) Animal treatment Male guinea-pigs weighing about 200 g were used The soleus and the EDL muscles were dissected out under pentobarbitone anesthesia and mounted in organ baths containing oxygenated Krebs solution at 37° Subtetanic contractions were evoked by transmural field stimulation every 15 s throughout the experiment (Waldeck 1976 Holmberg & Waldeck 1977)

The muscle preparations were allowed to stabilize for 1 h after which propranolol (0 03 μmol/l) was added followed 10 min later by terbutaline (0 3 μmol/l). After another 30 min the stimulation was stopped and the muscles were quickly removed wiped off and dropped into liquid nitrogen. Muscles treated with either propranolol or terbutaline and untreated muscles (controls) were run in parallel In some control expts the stimulation was stopped 30 min before the end of the expt (resting muscles). In order to make comparisons with the metabolic state under in-vivo conditions, some muscles were dropped into liquid nitrogen immediately after they had been dissected out. The average weight ± S D was 86 ± 30 mg for the soleus and 124 ± 2 mg for the EDL muscles were pooled 3 by 3 and stored in liquid n or packed in dry ice until the biochemical analysis

(b) Analytical methods The frozen tissue homogenized and treated with 0.6 mol/l HClO as previously described (Karlsson et al 1975) The perchloric extract obtained was analyzed for adenosine-triphosphate (ATP) (Lamprecht & Trautschold 1962), adenosine diphosphate (ADP) and adenosine-monophosphate (Adam 1962) creatinephosphate (CP) (Lamprecht Stein 1962) creatine (C) (Bernt et al 196.) and lactate pyruvate (Hohorst et al 1959) The content of c AMP analyzed by means of a protein-binding assay (The clic AMP Assay The Radiochemical Centre Amersham England Jan 1975)

(c) Statistical analysis Statistical analysis was performed using analysis of variance followed by the t Values of $P<0.05$ were considered statistically significant

RESULTS

(a) Functional effects

In the soleus muscle terbutaline (0 3 μmol/l) depressed the subtetanic contractions by about ($P<0.001$) (Fig 1) Propranolol (0 03 μmol/l) had no significant effect per se but attenuated the effect of terbutaline ($P<0.005$) In the EDL the force of subtetanic contractions increased by about 40% on the addition of terbutaline ($P<0.001$) This effect was completely blocked by propranolol ($P<0.0$ which by itself was devoid of effect compared to the control expt

4 The level of c-AMP in the resting and orking soleus and EDL muscles and the effect of *anolol (0 03 μmol/l) and terbutaline (0.3 μmol/l)*

S E. in μmol/kg wet weight and the number of determinations, each based on 3 pooled muscles. For further see legend to Fig. 1 and the methods section

	Resting muscles		Working muscles, in vitro			
le	In vivo	In vitro	Control	Propranolol	Terbutaline	Propr + Terb
s	3 7±1.0 (4)	0 50±0 07 (3)	0 85±0 30 (4)	0.49±0 02 (2)	1.22±0.30 (3)	0.81_0 08 (5)
	2.3±0 2 (5)	0.24±0 02 (3)	0.27±0 03 (5)	0 34±0 03 (4)	0.50±0 01 (4)	0.36±0 05 (5)

AMP being 7 10 times higher in vivo than in) ($P<0$ 001) The level of c AMP was 2 3 times er in the soleus than in the EDL for the working rols ($P<0$ 001) and for resting muscles 0 025) (Table 4)

erbutaline doubled the amount of c-AMP in the ($P<0$ 01) and this effect was inhibited by ranolol The results for the soleus muscle were e scattered but muscles treated with terbutaline wed a higher level of c AMP than those treated propranolol alone ($P<0.01$).

CUSSION

findings in this study show that the energy state he isolated soleus and EDL muscles is very le and does not significantly differ from the mal state in vivo This situation is based on the ity of the adenylate kinase and creatine phos-kinase reactions to maintain the involved reac s at concentrations close to equilibrium. For mple the mass action ratio of the isolated ylate kinase (Egglestone & Hems 1952) is in range 0 3–0 7 Although the ratios tended to be placed in the direction of ATP-breakdown in vit his displacement was close to the normal range r transmural field stimulation tended to improve energy state in so far as the mass action ratios e in the direction of ATP-synthesis The mus-movements may have a beneficial effect on the usion of substrates (cf Waldeck 1976) Although muscle movement per se may improve the rate diffusion of substrates and oxygen, the demand substrate and oxygen for ATP-synthesis is in-sed during muscle work. The increase in the tate/pyruvate ratio in the working soleus muscle icates slightly anaerobic conditions which may due to a limited supply of oxygen. This effect is bably a normal adaptive phenomenon during work rather than a difference between in-vivo and in-vitro conditions.

The energy and the redox state of the resting or working muscles in vitro compared with in vivo is thus stable It may therefore be assumed that the diffusion of substrates and oxygen from the medium is sufficient to maintain a relatively normal metabolism Similar conclusions have been drawn from experiments on isolated soleus and EDL mus cle from the rat (Chandry & Gould 1969 Partington & Wales 1976).

The effects of terbutaline on subtetanic contrac tions of the isolated soleus and EDL muscles were similar to those described previously (Waldeck 1977 Holmberg & Waldeck 1977) and they were blocked by propranolol There were no consistent changes in the energy or the redox state after treat ment with terbutaline or propranolol or both A slight reduction in CP level during terbutaline treatment was however observed in EDL. A simi-lar change in CP level during β-receptor stimulation with adrenaline was previously demonstrated in the vastus muscle of the rat in vivo (Fellenius et al 1979b) without any corresponding changes in the soleus content of CP This indicates that there may be a quantitative difference in the β-adrenergic con-trol of energy supply in the form of CP in the slow contracting soleus muscle compared with the fast EDL or vastus muscles (cf Fellenius et al 1980) The effect of terbutaline on CP did not significantly influence the energy state Thus the effects on the energy metabolism do not seem to cause changes in muscle contraction during treatment with β-recep-tor active compounds (cf Bowman & Nott 1969) No change in CP level was observed in the soleus after drug treatment in spite of pronounced effects on contractility

The effect of terbutaline on the subtetanic con-traction was accompanied by an elevation of the

Table 3 *The redox state in the resting and working soleus and EDL muscles and the effect of pro- (0 03 μmol/l) and terbutaline (0.3 μmol/l)*

Means in mmol/kg wet weight and the mean ratios respectively the S E. and the number of determinations (ea) on 3 pooled muscles For further details see legend to Fig 1 and the methods section

Muscle and variable	Resting muscles		Working muscles In vitro			
	In vivo	In vitro	Control	Propranolol	Terbutaline	Prop +T
Soleus						
Lactate	1 8 ±0.5 (5)	2.9 ±0.3 (4)	5 3 ±0 9 (4)	4 0 ±0 7 (4)	4 7 ±1 0 (4)	3 4 +[illegible]
Pyruvate	0 15±0 01 (5)	0 13±0 02 (4)	0 13±0.01 (4)	0 1 ±0 0 (4)	0 19±0 04 (4)	0 11+[illegible]
Lact./Pyr	1 ±3 (5)	23±4 (4)	40±7 (4)	38±1 (4)	26±3 (4)	35 [illegible]
EDL						
Lactate	4 9 ±0 8 (5)	4.3 ±1 0 (4)	5 6 ±0 4 (5)	5 4 ±0 8 (5)	6.5±0 6 (4)	5 0 +[illegible]
Pyruvate	0 22±0 05 (5)	0 11±0 01 (4)	0 1 ±0 0 (5)	0 10±0 02 (5)	0 16±0 0 (4)	0.14±[illegible]
Lact./Pyr	5±5 (5)	41±10 (4)	49±6 (5)	58±10 (5)	45±9 (4)	46±3 [illegible]

statistical significance was not attained The energy charge (EC) reflecting the ratio [ATP+1/2ADP] [ATP+ADP+AMP]$^{-1}$ (Atkinson 1968) remained very constant in all expts but it was in general about 2% higher in the EDL than in the soleus muscle ($P<0\ 001$) Most of the changes of the compounds involved in energy metabolism seen in the in-vitro preparation were restored to the in vivo levels after transmural electric stimulation i e in the working muscle Thus the increases in AMP ($P<0\ 05$) and C ($P<0\ 05$) in the soleus muscle during work compared to resting control muscles were statistically significant

When the soleus muscle was compared with the EDL in e g the working control mucles it appeared that the levels of ATP and CP were about 50% higher in the fast-contracting EDL than in the slow-contracting soleus ($P<0\ 001$) The level of AMP in the soleus was twice that found in the EDL ($P<0\ 025$) while the level of C showed a difference in the opposite direction ($P<0\ 05$)

(c) *Effects of terbutaline and propranolol on the energy state*

Propranolol and terbutaline given separately or together had no significant effects on the levels of ATP or ADP either in the soleus or in the EDL muscle (Table 1) AMP in the soleus however was lower in the drug-treated than in the control muscles ($P<0\ 05$) The level of CP in the EDL was reduced by about 20% after terbutaline ($P<0\ 005$) but no statistically significant changes in CP were induced in the soleus by either propranolol or terbutaline Nor did these drugs induce any ch[...] the level of C

(d) *The redox state in resting and working control muscles*

In general the lactate levels tended to be some[...] higher in the EDL compared to the soleus [...] in resting muscles both in vivo and in vitro (T[...] 3) although this effect was statistically signif[...] ($P<0\ 005$) for the "in vivo" state only Since py[...] vate did not change significantly it follows that [...] lactate/pyruvate ratio was in general higher i[...] EDL than in the soleus ($P<0\ 005$) There wa[...] statistically significant difference in the la[...] pyruvate ratio between the in-vivo and resting [...] vitro muscles

The lactate level in the soleus doubled d[...] work ($P<0\ 025$) but no such effect was [...] the EDL Since pyruvate was relatively cons[...] the lactate/pyruvate level was increased in the [...] eus muscle during work

(e) *Effects of terbutaline and propranolol on the redox state*

There were no drug-induced effects on the lev[...] either lactate or pyruvate or on the ratio betw[...] the two (Table 3)

(f) *Changes in levels of c AMP*

Owing to the wide range in the c AMP data statistical analysis was preceded by logarit[...] transformation The most marked difference [...] served was that between resting muscles the [...]

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level of c AMP in the muscle Accumulation of c AMP after β-adrenoceptor stimulation has previously been demonstrated in both slow and fast contracting skeletal muscle in vivo (Sullivan & Zaimis 1973) and in vitro (Al-Jeboory & Marshall 1978) although there are certain quantitative differences In our in vivo expts we found a much higher level of c AMP than normal However after stabilization in vitro our values averaged 0 5 μmol/kg (cf Steiner et al 1972)

We believe that the stress during the dissection procedure may have caused an increase in c AMP Al-Jeboory & Marshall (1978) reported a 3 times higher control level We have no explanation for this difference but it should be noted that in these authors experiments the concentration of agonist needed to elevate the c AMP level was two orders of magnitude higher than it was in our experiments although they used salbutamol which is more potent than terbutaline (Olsson et al 1979)

In summary our study lends no support to the view that the functional effects of β-adrenoceptor stimulation are primarily due to changes in the redox and energy state On the other hand c AMP appears to be a mediator as has been proposed by Bowman & Nott (1969 and 1974) Recent studies suggest that secondary to the changes in c AMP there may be changes in the K /Na balance ultimately leading to changes in the intracellular movement of Ca (Holmberg & Waldeck 1980)

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Physiol Scand 1980 109: 97-105

eraction of Substance P with dispersed pancreatic .nar cells from the guinea pig ıding of radioiodinated peptide[1]

ıS SJÖDIN ERNST BRODIN GÖRAN NILSSON and THOMAS P CONLON

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SJÖDIN L, BRODIN E, NILSSON G & CONLON T P Interaction of substance P (SP) with isolated acinar cells from the guinea-pig pancreas. Binding of radioiodinated peptide Acta Physiol Scand 1980 109: 97–105 Received 24 Sept. 1979 ISSN 0001-6772. Section on Pharmacology Department of Drugs, National Board of Health and Welfare, Uppsala, and Department of Pharmacology Karolinska Institutet Stockholm, Sweden

Tyr[8] SP has been radioactively labeled with ^{125}I to specific activity of 200 μCi/μg. This tracer has been found to be rapidly and reversibly bound in a specific way to dispersed pancreatic acinar cells. The specific binding is saturable and dependent on incubation temperature. At 37°C ^{125}I-tyr[8]-SP is bound to less degree than at lower temperatures since the peptide is rapidly degraded at 37°C Native SP inhibits binding of tracer Results have been analyzed according to Scatchard and suggest 2 functionally distinct types of binding sites small number of sites with high affinity and a larger number of sites with low affinity for SP SP-sites also bind eledoisin and physalaemin which are peptides similar to SP in terms of chemical structure and biological activities. The concentration range in which SP physalaemin, and eledoisin inhibit binding of ^{125}I-tyr[8]-SP correlates well with the range in which these peptides stimulate biochemical processes like outflux of ^{45}Ca, accumulation of cyclic GMP and release of amylase from acinar cells.

Key words: Acinar cells, amylase calcium, cyclic GMP eledoisin, pancreas physalaemin substance P

)31 von Euler and Gaddum discovered the pres- of a biologically highly active *principle* in ex ı from brain and gut that subsequently became vn as substance P (SP). Forty years later it chemically characterized as an undecapeptide ng et al 1970) and subsequently synthetized pear et al. 1971)) Using radioimmunoassay tance P-like immunoreactivity (SPLI) has been onstrated in extracts of a great number of es including the pancreas (Nilsson & Brodin) Immunohistochemical studies have provided hological evidences for the existence of SPLI lized to nervous structures in the pancreas son et al. 1976, Larsson & Rehfeld 1979) In investigations indicate that exogenously ad- stered SP evokes secretion of amylase from the reas (Starke et al 1968 Thulin & Holm 1977) onsidering the immunochemical and immuno- logical demonstrations of SPLI in the pancreas the ability of SP to functionally influence the exocrine pancreatic cells we decided to investigate whether binding sites capable of specific binding of SP are present on dispersed pancreatic acinar cells from guinea pigs and whether such binding may be correlated to biochemical effects in these cells. The present paper deals with characteristics of binding of SP to dispersed pancreatic acinar cells. In an accompanying paper we study some biochemical *effects* of SP on acinar cells.

MATERIALS AND METHODS

Male guinea pigs (150–200 g) were obtained from Harry Jansson, Märsta, Sweden. Crude collagenase (E.C. 3.4.24.3) and crude hyaluronidase (E.C. 3.2.1.35) and purified soy-bean trypsin inhibitor were purchased from Sigma Chemical Co. St Louis, M Carrier-free N ^{125}I (100 μCi/μl) in 0.1 N Na OH was obtained from the

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[1] Part of this investigation were presented at XVI Scandinavian Congress of Physiology and Pharmacology Oslo 1979

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Radiochemical Centre Amersham England Chloramine T was purchased from Eastman Kodak Co Rochester N Y sodium metabisulfite from J T Baker Deventer Holland bovine serum albumin from Miles Laboratories Inc. Elkhart Indiana. All other chemicals were of the highest grade commercially available

Synthetic bovine SP was obtained from Peninsula Laboratories Inc San Carlos Calif

For iodination purposes an analogue was used in which the phe[8] residue had been substituted by tyr This analogue was a gift from Dr Karl Folkers Inst for Biomed Res Univ of Texas Austin Texas Synthetic bombesin caerulein eledoisin and physalaemin were donated by Dr Roberto de Castiglione Farmitalia S p A Milano Italy

Highly purified natural porcine cholecystokinin and natural porcine secretin and natural vasoactive intestinal polypeptide (VIP) were gifts from Dr Viktor Mutt GIH research unit Karolinska Institutet Stockholm Sweden

Highly purified natural porcine gastric inhibitory polypeptide (GIP) was donated by Dr John C Brown Dept of Physiology Univ of British Columbia, Vancouver B C

Preparation of ^{125}I tyr^8-SP (labelled SP)

Tyr^8-SP was iodinated according to the chloramine T method (Hunter & Greenwood 1962) The procedure was performed at room temperature in a 1 ×70 mm polystyrene tube 300 μCi Na ^{125}I in 20 μl of 0 25 M sodium phosphate buffer pH 7 5 was reacted with 5 μg (3 7 nmol) tyr^{8}SP dissolved in 50 μl 0 15 M sodium chloride by addition of 10 μg (35 5 nmol) of chloramine T in a volume of 10 μl After 30 s the reaction was terminated by addition of 20 μg (105.2 nmol) of metabisulfite dissolved in a volume of 20 μl (Both the chloramine T and the metabisulfite were dissolved in 0 25 M phosphate buffer pH 7.5) The reaction volume was then diluted with 1 0 ml 0 15 M sodium chloride containing 50 mg (740 nmol) of bovine serum albumin and transferred to another testtube containing 10 mg of microfine precipitated silica (Quso G 32, Philadelphia Quartz Co Philadelphia) Labelled SP was bound to the silica and later eluted with a mixture of acetone and acetic acid as described by Yalow & Berson (1966) for parathyroid hormone

After this preliminary purification the iodinated material was subjected to gel filtration on Sephadex G 25 superfine (9×600 mm) at 4°C using 0 05 M veronal buffer pH 8 6 with 4 mg (59 nmol) of bovine serum albumin per ml as eluent. Fractions (0 85 ml) were collected and counted directly in a gamma counter The fractions giving the highest degree of binding in our RIA system (Nilsson et al 1977) were pooled and used for the receptor-binding experiments The specific activity of the ^{125}I-tyr^8 SP as calculated by comparing self-displacement curves of tracer with ordinary standard curves in the RIA system was 200 μCi/μg.

Preparation of dispersed pancreatic acinar cells

Guinea pigs were deprived of food overnight and killed by a blow to the head The pancreas was dissected free from fat and mesentery and dispersed acinar cells were prepared as described by Gardner et al (1975) using minor modifications of the technique of Amsterdam & Jamieson (1972, 1974) Cells were suspended in Krebs-Ringer bicarbonate (pH 7 4) equilibrated with 95 % O_2 and 5 % CO_2 containing L-aminoacid supplement (Eagle 1959), soybean trypsin inhibitor per ml 15 mM glucose (w/v) bovine serum albumin and 0.5 mM calcium The cell concentration was 12×10^6±0 8×10^6 cells/ml

Binding assay

Cells were incubated with ^{125}I-tyr^8-SP at a concentration of 5×10^{-} M at various temperatures (4 12, 22 or 37 At appropriate times after addition of ^{125}I-tyr^8 SP duplicate or triplicate 100 μl samples were layered over 300 μl Krebs-Ringer bicarbonate buffer in microfuge tubes polypropylene The cells were centrifuged at 10 000 g 15 s in a Beckman model B microfuge The pellets washed a total of 4 times The microfuge tubes containing washed pellets were placed in a Packard 5100 gamma counter Duplicate samples were also taken from the incubation mixture containing both cells and medium placed in the counter

The obtained values were used to calculate bound ^{125}I tyr^8 SP as the percentage of added ^{125}I-tyr^8 SP binding of radioactive SP in the presence of 10^{-6} M cold referred to as nonspecific binding

This value was on average equal to 38 % ± 3 % of the total binding observed at equilibrium conditions

Dissociation of bound labelled SP

Cells were preincubated with ^{125}I-tyr^8-SP for 60 min 22°C as described above (Binding assay). After centrifugation (2 min 500×g) the supernatant was aspirated and cells resuspended in fresh buffer with or without unlabelled SP (10^{-6} M) and a second incubation started at 12 or 37°C Samples were taken from the incubation medium immediately and after 10 20 30 45 and 60 min and binding was determined Total radioactivity remaining in medium after resuspension was less than 3 % of the original value

Functional alteration (deteriorated binding) of labelled SP following incubation with acinar cells

^{125}I-tyr^8 SP was incubated with cells for various times at different temperatures (30 min at 37°C 60 min at and 90 min at 12°C) Cells were then centrifuged for microfuge tubes at 10 000×g The supernatant was aspirated and added to fresh cells in such numbers that concentration remained unchanged Binding of labelled to cells was then determined at 22°C as described (Binding assay) after various incubation times and expressed as percent of maximal uptake of labelled SP in incubations in which fresh tracer had been added

Chemical alteration (chromatographic behaviour) of labelled SP following incubation with acinar cells

^{125}I tyr^8 SP was incubated in buffer solution with or without cells at different temperatures (12 22 and 37 described above and samples (300 μl) taken from the incubation mixture at various times (1 120 min) Samples were spun (15 s) in a microfuge the supernatants aspirated and placed in plastic tubes and then immediately frozen in an acetone-dry ice bath This procedure required

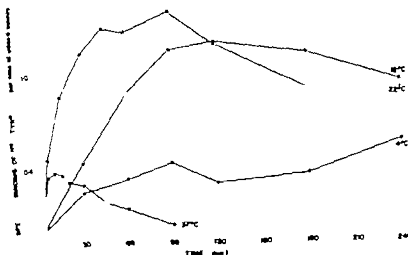


Fig 1 Time course of specific binding of ^{125}I-tyr^8 SP to pancreatic acinar cell as function of incubation temperature Cells were incubated with 5 10⁻ M ^{125}I-tyr^8 SP Each point represents the mean of results from 4–6 xpts.

...mately 1 min Samples were then stored at −20°C chromatographed on Sephadex G-25 superfine col... using the same parameters as described under Pre... ...ion of ^{125}I-tyr^8 SP The extent of degradation was ...timated from the elution curves of radioactivity ...andard to which the samples were compared con... of labelled SP taken directly from storage and di... to ...ular concentration and volume as the incu... samples before being chromatographed on the same ...m.

...ULTS

...ime cours of specific binding of labelled SP ...persed pancreatic acinar cells (Fig 1)

...temperature of 37°C specific binding of ^{125}I-... SP was maximal after 10 min incubation and ...decreased gradually to 10% of the maximal ...e after 90 min. At 22°C binding was as rapid ...ally as at 37°C However during incubation for ...to 45 min binding increased further after which ...e it levelled off at a degree of 1.3% specific bind... After 90 min of incubation at this temperature ...cific binding tended to slowly decrease A reduc... ...of the incubation temperature to 12°C de... ...ased the binding rate but the maximal specific ...ding was not significantly different from that ...ined at 22°C Incubation at 4°C further reduced ...rate of binding and the highest degree of speci... ...binding obtained during 4 hours of incubation ...at this temperature around 0.6%

...When the pH of the incubation medium was varied within the range of pH 6.6–8 4 binding was found to be maximal at pH 7.8 Results are not shown

B Time course of dissociation of bound labelled SP (Fig 2)

The dissociation rate was found to be temperature dependent over the temperature range tested (12–37°C) being highest at 37°C. At 22°C the initial rate was approximately 2%/min Addition of nonradioactive bovine synthetic SP (10^{-6} M) did not affect the rate of dissociation at any temperature

C F nctional alteration (deteriorated binding) of labelled SP following inc bation with acinar cells (Fig 3)

The low degree of specific binding of ^{125}I-tyr^8 SP at 37°C suggested to us that SP was rapidly broken down in our system at that temperature To study the functional alteration of SP during incubation with acinar cells, binding experiments were per formed after preincubation of ^{125}I-tyr^8 SP with cells at different temperatures and for various periods of times (see methods) In all cases pre incubated radioligand gave lower maximal specific binding compared to what was obtained with fresh labelled SP The impairment of binding was however much more pronounced following incubation at 37°C than at 22° or 1?°C, maximal binding being 30% 50% and 58% of maximal binding of fresh

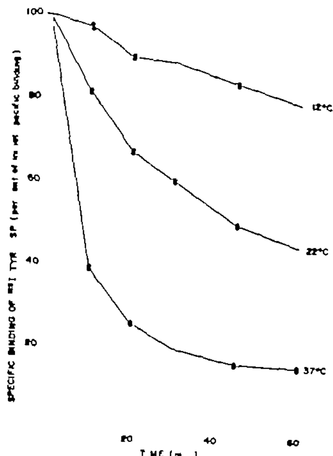


Fig 2 Effects of temperature dilution and dilution with addition of native SP (10^{-6} M) on dissociation of specifical ly bound ^{125}I-tyr[8] SP from pancreatic acinar cells Cells were preincubated with ^{125}I-tyr[8]-SP (5×10⁻ M) for 60 min at 22°C. Cells were then spun at 500×g for 2 min Super natant was aspirated and cells resuspended in fresh buffer of indicated temperature with and without native SP (10^{-6} M) Graph shows the percentage of ^{125}I tyr[8]-SP specifical ly bound to the cells immediately after resuspension which remain on the cells at indicated times thereafter Each point represents the mean of 3 separate expts Closed circles (●) denote resuspension in buffer alone and open circles (O) denote resuspension in buffer with 10^{-6} M native SP

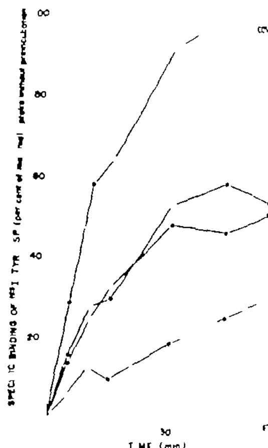


Fig 3 Determination of bound ^{125}I tyr[8]-SP after pr bation at various temperatures with pancreatic cells Cells were incubated for 30 min at 37°C 60 22°C and 90 min at 12°C with ^{125}I-tyr[8]-SP (5×10 Cells were then spun in a microfuge for 15 s (100 The supernatant was aspirated and added to fresh whose uptake of tracer was determined at 22°C cated times Results are expressed as precent of uptake of ^{125}I-tyr[8] SP in control incubations in which tracer had been added to cells Each point repr mean of 3–4 expts

tracer respectively in spite of shorter preincubation times at the higher temperatures

D *Chemical alteration (chromatographical behaviour) of labelled SP following incubation with cells (Figs 4 and 5)*

In order to further study the possible enzymatic degradation of ^{125}I tyr[8] SP during incubation buffer solutions were incubated with and without cells at different temperatures and for various times The degree of chemical alteration of the tracer was then estimated from the elution patterns of radioactivity following gel filtration on Sephadex G-25 superfine The fraction of radioactivity eluting in the peak corresponding to intact labelled SP was moder reduced with increasing incubation time at and 22°C while incubation at 37°C produced a shift of radioactivity to the right in the el pattern indicating a rapid destruction of labelled at this temperature Thus the results of the col experiments showed the same general tendency the results of the rebinding experiments der strating a clear temperature dependence of degradation rate

E *Inhibition of binding of labelled SP by synthetic SP and structurally related peptides (Fig 6)*

The ability of various concentrations of synth SP eledoisin and physal inhibit binding

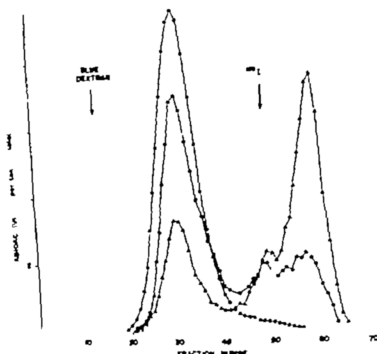


Fig. 4. Deterioration of free ^{125}I-tyr^{8} SP. Cells were incubated with ^{125}I-tyr^{8}-SP for various times at different temperatures. A 300 µl sample was taken and spun in the microfuge (10 000×g) for 15 s. The supernatant was aspirated and stored at −20°C until put on Sephadex G-25 superfine column (9×600 mm) and run with veronal buffer. The graph shows the elution pattern for an unincubated standard preparation of ^{125}I-tyr^{8} SP (closed circles) and the supernatant from cell suspension incubated with ^{125}I-tyr^{8} SP for 1 h at 12°C (open circles) or 37°C (triangles).

r^{8} SP to acinar cells was examined. Incuba-with synthetic SP were carried out at 22°C for n or at 12°C for 90 min. The results were tially the same at both temperatures. Inhibi-f specific binding of labelled SP could be de-l at 1×10^{-10} M SP was halfmaximal at 10^{-9} M round 70% at 2×10^{-9} M. At 3×10^{-6} M specific ng was about 5% of control binding. The data also plotted according to Scatchard (1949). plot displayed an upward concavity and the r part of the curve could be described by 2 ght lines. This suggests to us that there may be distinct types of binding sites for SP on the ar cells. We have calculated the apparent affi-and capacity for each type from results of 11 rate experiments at 22°C and 5 experiments at (Table 1). Similar results were obtained for incubation temperatures indicating that there approximately 300 high affinity sites and 2 000 sites of the low affinity type per cell. Half of the high affinity sites will be occupied at a SP-concentration of 9×10^{-10} M and 50% of the low-affinity sites at about 2–3×10^{-8} M. These figures must be interpreted with caution, since our experimental conditions do not meet all criteria required for this type of analysis (Rodbard 1973, Boeynaems & Dumont 1975). Eledoisin and physalaemin which have structural similarities with SP also inhibited binding of ^{125}I-tyr^{8} SP to the acinar cells. Only relatively high concentrations of eledoisin and physalaemin (10^{-6} M) did inhibit binding.

Some other peptides that have earlier been shown to have biological effects on pancreatic acinar cells were also tested for ability to interfere with binding. However, secretin at a concentration of 5×10^{-6} M, GIP at 1×10^{-6} M, caerulein, bombesin and VIP at 10^{-6} M did not significantly affect binding of ^{125}I-tyr^{8}-SP.

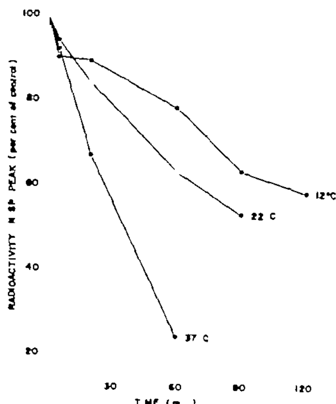


Fig 5 Deterioration of free ^{125}I tyr^8 SP Cells were incubated with ^{125}I-tyr^8 SP for indicated times at indicated temperatures A 300 μl sample was taken and spun in the microfuge (10 000×g) for 15 s The supernatant was aspirated and stored in a deepfreeze until put on a Sephadex G-25 superfine column (9×600 mm) and run with veronal buffer The graph shows percent of radioactivity remaining in initial peak representing intact ^{125}I tyr^8-SP In control experiments with incubation of ^{125}I-tyr^8-SP in buffer without cells there was no significant degradation of SP at 12°C and 22°C while there was approximately 20% degradation at 37°C after 1 h

DISCUSSION

Specific binding of radioactively labelled SP or tyr^8 SP has earlier been demonstrated to a crude synaptic membrane preparation from rabbit brain (Nakata et al 1978) and to isolated synaptic vesicles from rat brain (Mayer et al 1979)

The present paper demonstrates binding of labelled SP to pancreatic acinar cells and further illustrates that the binding is rapid specific reversible and dependent on incubation temperature The relatively low degree of maximal specific binding obtained at 37°C incubation temperature compared to what was obtained at lower temperatures seems to be explained by a SP degrading activity present in the pancreatic acinar cells Both the functional and chemical alteration of labelled SP following in

Table 1 *Characteristics of binding sites [illegible] tyr^8 SP on dispersed guinea-pig pancreatic [illegible] cells*

$S_{0.5}$ is concentration (M) of SP required to occupy [illegible] the indicated type of ^{125}I-tyr^8 SP binding sites Th[illegible] represents means ± S E. from 10 separate exp[illegible] and 5 expts. at 12°C

	High affinity SP binding site	Low affin[illegible] SP binding si[illegible]
Number of sites per cell	260±50 22°	2200±3[illegible]
	360±60 12°	1900±590 [illegible]
$S_{0.5}$	8.8 (±1.5)×10[illegible] 22°	3 1 (±0 6) [illegible]
	9 5 (±1 0)×10 [illegible]	2.1 (±0 7)×[illegible]

cubation with cells were studied and in b[illegible] a clear temperature dependance was ob[illegible] speaking in favour of an enzymatic nature of t[illegible] degrading activity The occurrence of SP-in[illegible] ing enzymes has formerly been shown in [illegible] organs and tissues including for examp[illegible] (Benuck & Marks 1975) kidney (Ward & [illegible] 1978) endothelial cells (Johnson & Erdös 1[illegible] blood (Bury & Mashford 1977) In an att[illegible] inhibit the SP inactivation during the bind[illegible] periments aprotinin (Trasylol® 500 KIE/[illegible] bacitracin (50 μg/ml) were included in the [illegible] tion medium in some experiments. Aprotin[illegible] bacitracin in these concentrations have [illegible] systems been shown to inhibit SP-deg[illegible] (Johnson & Erdös 1977 Cuello et al 197[illegible] beck et al 1978) In our hands these measur[illegible] ineffective

Scatchard plots of ^{125}I tyr^8 SP binding at 12[illegible] 22°C were curvilinear with an upward conc[illegible] The curves could be fitted to two straight [illegible] This has been interpreted as indicating th[illegible] were two functionally distinct classes of SP [illegible] sites Since the rate of dissociation of [illegible] ^{125}I-tyr^8 SP was not altered by addition of un[illegible] our data do not appear to demonstrate neg[illegible] operativity (Kahn 1975 Boeynaems & D[illegible] 1975) We do not know if the two types of [illegible] sites exist on all cells or if there are two popul[illegible] of cells with different types of binding sites.

Physalaemin and eledoisin are two peptide[illegible] chemical and biological characteristics simila[illegible] which have been isolated from the skin of [illegible] bias (physalaemin) and from the posterior s[illegible] gland of a Mediterr[illegible] octopod (eledois[illegible]

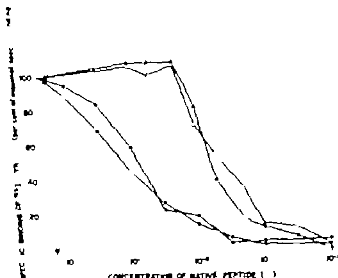


Fig 6 Ability of native SP eledoisin, and physalaemin to inhibit binding of ^{125}I-tyr^8-SP (5 10^{-} M) to pancreatic acinar cells. Cells were incubated with native SP either for 60 min at 22°C (open circles) or 60 or 90 min at 12°C (closed circles). In experiments with eledoisin or physalaemin incubation were carried out at 22°C for 60 min. Open triangles denote experiments with physalaemin and filled triangles experiments with eledoisin. Binding of ^{125}I-tyr^8 SP is expressed as percentage of radioactivity bound specifically in the absence of added nonradioactive peptide Each point represents the mean of at least 6 separate expts

ier group (Anastasi & Erspamer 196.., lertaccini et al 1965).

ted pancreatic acinar cells have been show oond to incubations with physalaemin and in with elevated calcium outflux. Increased r accumulation of cyclic GMP and increased on of amylase (May et al 1978). Our present show that binding sites for SP on the acinar also bind these two peptides. The reported g data for eledoisin and physalaemin also cor with reported biochemical effects of these les on acinar cells (May et al. 1978). Thus it een found that eledoisin and physalaemin in a ntration of 3 10^{-8} M significantly stimulate ar accumulation of cyclic GMP This con ation of the peptides produced 60–70% inhibi of ^{125}I-tyr^8 SP binding.

some preliminary experiments we have ed inhibition of ^{125}I-tyr^8 SP by addition of un- led tyr^8-SP instead of native SP In contrast to er et al. (1979) who found that tyr^8 SP did not fere with binding of ^{125}I-tyr^8 SP to synaptic les we hav found that tyr^8 SP inhibits binding I-tyr^8 SP to pancreatic acinar cells to degree ar to that obtained with native SP

The results of our study of the biochemical ef fects of SP on calcium outflux from acinar cells cellular accumulation of cyclic GMP and stimulation of amylase release are reported as an accompanying paper Effects on these parameters are seen with concentrations of SP which are in the same range as those which inhibit binding of ^{125}I tyr^8 SP Maximal effects on cyclic GMP accumulation and amylase release are seen at a concentration of 3×10^{-8} M SP (Sjödin et al. 1979)

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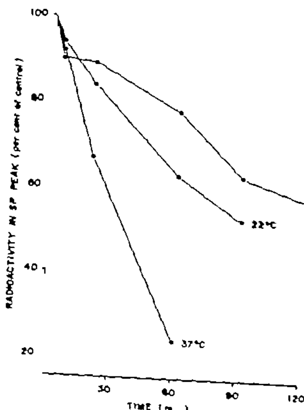


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Table 1 Characteristics of binding sites ... tyr^8-SP on dispersed guinea pig pancreatic cells

$S_{..}$ is concentration (M) of SP required to occupy ... the indicated type of ^{125}I tyr^8 SP binding sites ... represents means ± S E. from 10 separate expts ... and 5 expts at 17°C

	High affinity SP binding site	Low affinity SP binding site
Number of sites per cell	260±50 ...	200±19...
	360±60 ...	1 500±50
S	8.8 (±1.5)×10...	3.1 (±0.9)...
	9.5 (±1 0)×10...	...1 (±0.7)...

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SJÖDIN THOMAS P CONLON CARIN GUSTAVSON and KRISTINA UDDHOLM

SJÖDIN L., CONLON T P GUSTAVSON C. & UDDHOLM, K.. Interaction of Substance P (SP) with dispersed pancreatic acinar cells from the guinea pig. Stimulation of calcium outflux, accumulation of cyclic GMP and amylase release Acta Physiol Scand 1980, 109 107–110. Received 24 Sept. 1979 ISSN 0001-6772. Section on Pharmacology Department of Drugs, National Board of Health and Welfare, Uppsala, and Department of Pharmacology Karolinska Institutet, Stockholm, Sweden.

In dispersed acinar cells from the guinea pig pancreas, substance P (SP) was found to stimulate outflux of ^{45}Ca, cellular accumulation of cyclic GMP and release of amylase Maximal effects on accumulation of cyclic GMP and release of amylase were obtained with 3 10^{-8} M of SP 10^{-7} M of SP caused maximal outflux of ^{45}Ca. These effects corresponded to 30–50% of the maximal effects obtained with caerulein, cholecystokinin-like decapeptide The concentrations of SP required for stimulation of ^{45}Ca outflux, accumulation of cyclic GMP and release of amylase corresponded well with those which affect binding of ^{125}I-tyr^{8} SP to pancreatic acinar cells.

Key words Acinar cells, amylase caerulein, calcium, cyclic GMP pancreas, substance P

ave shown in an accompanying paper that yr^{8} substance P (SP) a radioactively labelled ative of SP is specifically bound to dispersed reatic acinar cells from the guinea pig. Others shown that substance P in high concentrations ase cyclic GMP concentrations in pancreatic es from the guinea pig (Albano Bhoola & ey 1978) Major stimulants of enzyme secre from the pancreas like cholecystokinin, and lcholine have been thought to act upon the r cells through stimulation of calcium outflux accumulation of cyclic GMP (Gardner et al. Christophe et al 1976, Sjödin & Gardner) SP has also been reported to stimulate panc enzyme secretion in vivo (Starke et al 1968 in & Holm 1977). We have investigated the cts of SP on calcium outflux, accumulation of c GMP and amylase release from dispersed ar cells from the guinea pig pancreas and ex ned the correlation between those effects of SP binding of SP to the cells. We have compared effects of SP with those of a cholecystokinin-peptide caerulein.

MATERIALS AND METHODS

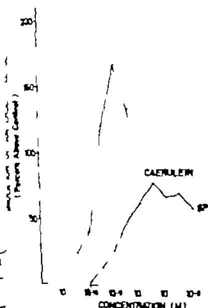
In addition to the peptides and reagents listed in the preceding paper (Sjödin et al. in press) ^{45}Ca (12.5 mCi per mg, ^{125}I-succinyl GMP TME antigen, ^{125}I-succinyl cyclic AMP TME antigen and cyclic GMP and AMP specific antisera were purchased from New England Nuclear Corp. Boston, Mass. Hepes (N 2-hydroxyethyl-piperazine-N-ethanesulfonic acid) from Kebo, Stockholm and Phadebas amylase test tablet from Pharmacia Fine Chemicals AB Uppsala, Sweden

Dispersed pancreatic acinar cells were prepared according to the technique of Amsterdam and Jamieson (1972, 1974 *b*). Unless otherwise specified cells (12 10^{6}±0 8 10^{6} cells/ml) were suspended in a standard incubation solution composed of Krebs-Ringer bicarbonate (pH 7 4) with L-amino acid supplement (Eagle 1959), 0 1 mg of soybean trypsin inhibitor per ml, 15 mM glucose 1% (w/v) albumin and 0.5 mM calcium, and gassed with 95% O_2 5% CO_2.

Outflux of ^{45}Ca Cellular loss of ^{45}Ca was measured as described by Gardner et al (1975) Cells were preincubated with ^{45}Ca for 60 min and the loss of cellular radioac

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Parts of this investigation were presented at XVI Scandinavian Congress of Physiology and Pharmacology Oulu 1979



...4. Effect of caerulein and substance P on release of ...se from dispersed pancreatic acinar cells. Acinar ...vere suspended in standard incubation solution in ... N-2-hydroxy-ethylpiperazine-N-ethanesulfonic ...(HEPES) replaced bicarbonate and which contained ...M calcium and 5 mM theophylline. Release of ...se was calculated as the difference in the percentage ...al cellular amylase which was present in the super... at the beginning and end of the 30-min incubation at ... Each point represents the mean of at least 18 ...ate incubations.

... 3). Increase in maximal release of amylase ...e basal was approximately 50% of the increase ...ced by a maximal dose of caerulein (Fig. 3)

...USSION

... cholecystokinin-related substances and cho...gic agents (Christophe et al. 1971 Sjödin & ...ner 1977), SP appears to stimulate amylase ...sion from pancreatic acinar cells by activation ...lcium outflux and accumulation of cyclic GMP ... efficacy of SP was relatively low. For calcium ...ux cellular accumulation of cyclic GMP and ...lase release maximal effects obtained with SP ... 30-50% of those with caerulein. The relative ...nstant relation between maximal effects of SP ... caerulein on different parameters involved in ...ar cell secretion suggests that they act through ...lar biochemical pathways. An alternative way ...timulation of pancreatic enzyme secretion may ...hrough accumulation of cyclic AMP. Amylase release induced by secretin and vasoactive intestinal polypeptide (VIP) appears to be mediated through cyclic AMP (Gardner, Conlon & Adams 1976, Robberecht, Conlon & Gardner 1976, Gardner & Jackson 1977). However, neither SP in the present study nor cholecystokinin (Gardner, Conlon & Adams 1976) stimulate cyclic AMP accumulation in acinar cells.

Our findings in the accompanying paper dealing with binding of ^{125}I-tyr^{8}-SP to acinar cells (Sjödin et al. in press) suggest that caerulein does not interact with the receptor for SP. Earlier findings indicate that cholecystokinin-like peptides and cholinergic agents act through different receptors on acinar cells (Gardner et al. 1975, Christophe et al., 1976). A third type of membrane receptors on acinar cells, specific for bombesin (Erspamer & Melchiorri 1975), has recently been reported (Jensen et al. 1978). Bombesin also increases calcium outflux, cyclic GMP and amylase secretion (May et al. 1978). However, bombesin according to our results (Sjödin et al. in press) does not interfere with binding of ^{125}I-tyr^{8} SP. The present study thus indicates that there is a fourth type of receptors affecting the same system. Our accompanying paper suggests that there is a high affinity and a low affinity binding site for SP. Occupation of the high affinity binding sites apparently contributes to a substantial extent to the subsequent biochemical effects.

May and co-workers (1978) found that the SP like peptides eledoisin and physalaemin stimulated calcium outflux, cellular cyclic GMP accumulation and amylase release. Since these peptides have affinity towards the SP binding sites, it is reasonable to assume that they exert their effects on outflux of calcium, accumulation of cyclic GMP and release of amylase by occupation of the same receptors as SP. This hypothesis is also supported by the observation that maximal effects on these parameters appear to be the same with SP, eledoisin and physalaemin.

To what extent SP plays a role in the physiological regulation of pancreatic exocrine secretion cannot be decided from the present experiments. Demonstrations of SP-like immunoreactivity in the pancreas (Larsson, Sundler & Håkansson 1976, Larsson & Rehfeld 1979) and stimulatory effects of SP on pancreatic enzyme secretion during in vitro (Albano, Bhoola & Harvey 1977) and in vivo conditions (Starke et al. 1968, Thulin & Holm 1977)

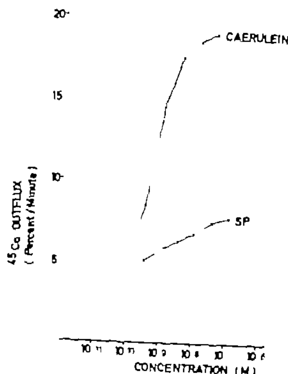


Fig. 1. Effect of caerulein and substance P on ^{45}Ca outflux from dispersed pancreatic acinar cells. Acinar cells were preincubated in standard incubation solution containing 0.5 mM ^{45}Ca for 60 min at 37°C. Appropriate concentrations of the indicated agents were added and ^{45}Ca outflux was calculated from the loss of the cellular ^{45}Ca during a 5-min incubation with 5 mM ethylenediaminotetraacetate (EDTA). Each point represents the mean of at least 11 separate incubations.

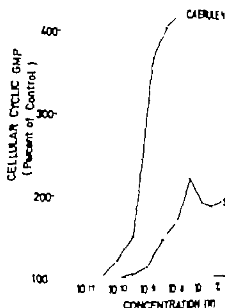


Fig. Effect of caerulein and substance P on cyclic GMP in dispersed pancreatic acinar cells ... were incubated at 37°C in standard incubation ... containing 0.5 mM calcium and 5 mM theophylline ... lar cyclic GMP was determined at the end of ... incubation with caerulein or 1 min with SP. Each ... represents the mean of at least 4 separate experiments.

tivity during a 5 min incubation period at 37°C with 5 mM ethylenediaminetetraacetate (EDTA) was determined.

Cellular cyclic GMP and AMP. Cellular cyclic GMP and AMP levels were measured by radioimmunoassay. Acinar cells were suspended in standard incubation solution containing 5 mM theophyllin and incubated at 37°C. Incubations were stopped by adding iced ethanol. The samples were centrifuged at 1000×g for 20 min. An aliquot of supernatant was acetylated by addition of triethylamine and acetic anhydride and then assayed for cyclic GMP or AMP as described by Harper & Brooker with some modifications (1975). Standards were prepared by adding known amounts of cyclic GMP or AMP to standard incubation solution containing 5 mM theophylline and processed the same way as the experimental samples.

Amylase release. For amylase release expts. acinar cells were suspended in buffer identical to the standard incubation solution except that the bicarbonate was replaced by an equimolar amount of HEPES (pH 7.4) and that it contained 5 mM theophylline. Gassing was performed with 100% O_2. After 0 and 30 min of incubation at 37°C an aliquot of cell suspension w s centrifuged at 10 000×g for 15 s using a Beckman Microfuge (Beckman Instruments, Inc. Spinco Division, Palo Alto, Calif.). An adequately diluted aliquot of supernatant was assayed for amylase activity by the method of Ceska, Birath ... (1969) using the Phadebas reagent as substrate. To ... ure total amylase present in the suspension, ... cell suspension was added to 10 volumes of ... containing 0.01 M sodium phosphate (pH 7.0), ... albumin and 0.1% (w/v) sodium dodecylsulfate, ... and assayed for amylase activity. The rate of ... release was taken as the difference between the ... present in the supernatant at the start of and after ... of incubation and was expressed as the percentage ... amylase activity.

RESULTS

Incubations with increasing concentrations of ... induced dose related enhancement of ^{45}Ca o... from acinar cells (Fig. 1). 3×10^{-10} M appe... produced a slight stimulation of calcium outflux ... a concentration of 10^{-7} M the rate of ^{45}Ca ... was only about 40% of that attained with $3\times10^{...}$ of caerulein (Fig. 1).

3×10^{-6} M of SP increased cellular accumul... of cyclic GMP to a maximum of about 40% of ... obtained with 10^{-8} M caerulein (Fig. 2). Neither ... nor caerulein had any significant effects on ... AMP levels (not shown).

Amylase release was augmented by SP ... reached a maximum at a co... tration of $3\times10^{...}$

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thod to improve the size uniformity of microspheres

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microspheres are used to measure blood he amount that may be injected is limited by fects, primarily a rise in arterial pressure. It is re desirable that the proportion of spheres tually get stuck in the capillaries is as large as le. Too small spheres, which pass through pillaries, as well as too large spheres which e held up in the arterioles should be avoided.

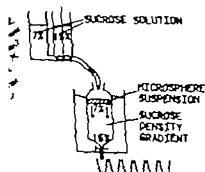
especially the case if blood flow is measured ll tissue segments since the spheres found e so few that the statistical variability of the becomes embarrassing. From this point of commercially available microspheres, in par the more inexpensive varieties, tend to have de size ranges. Also in developing automatic ds for sphere counting uniform sphere size e advantageous.

refore, a procedure to separate a batch of spheres into fractions with better size defini- ay be useful. Källskog et al. (1977) published such method where microspheres were ed to sediment in water. Here a modification s method is presented (Fig. 1). The essential es are that aggregation of the spheres is com- ed with dextran and Tween and that convection is diminished with the aid of a sucrose density gradient. In this way fractions with a standard deviation of size of approx. 1 µm may be achieved.

Materials and methods. 10 µm microspheres produced by New England Nuclear were used. These spheres are relatively inexpensive and do not aggregate much but vary much in size (Fig. 2).

The separation was carried out in Macrodex (Pharmacia) with 0.01% Tween-80 added. Macrodex being a medical product, is expensive but easily available. Any similar dextran preparation should be as useful.

Solutions of sucrose, 7% and 15% in Macrodex Tween were prepared and allowed to reach room temperature. With 200 ml each of these solutions, using standard equipment (two communicating vessels) a sucrose density gradient was laid in a 500 ml cylindrical separating funnel with 6 cm diameter kept thermostated in a water bath close to room temperature. The system is not extremely temperature-sensitive, and in a room where sudden draughts etc. can be eliminated, the water bath may not be necessary. Laying the gradient took about 1 h, and a further 30 min were allowed for stabilization.



The experimental equipment employed. The water surrounds the funnel where the separation takes The equipment at the left is used to make the ntration gradient.

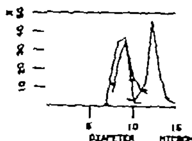


Fig. 2. The result of successful separation. Crosses show the composition of the original batch. Curves show the composition of 3 fractions. Distributions are founded on samples of size 100.

have suggested a physiological role for SP in the regulation of pancreatic exocrine secretion. The present study has shown specific binding sites for SP on pancreatic acinar cells and demonstrated a correlation between occupation of SP-binding sites and activation of amylase secretion. According to Larsson & Rehfeld (1979) most SP in the pancreas is located in peptidergic nerves which mainly innervate ganglia. They suggest that the ganglia represent an important physiological site of action in the pancreas for SP. The present results show that SP also acts directly on the acinar cells of the guinea pig.

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..nonstration of a proton gradient across the insulin ..nule membrane

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.. the last few years the existence of a proton-..cating mechanism in the chromaffin granule ..ane has been well established (Johnson et al ..) Proton translocation enables the granules ..ntain an internal pH below that of the sur..ng cytoplasm. One probable physiological ..on of the electrochemical gradient is to pro..nergy for the accumulation of amines within ..anules (Johnson et al 1978a) This mech.. for accumulation of amines in granules ..be a general phenomenon. Johnson et al ..) demonstrated the presence of a pH differ..n the serotonin granules of platelets and the .. to concentrate amines in secretory granules ..red by all cells producing polypeptide hor.. (Owman et al. 1973) I.. the latter types of the primary function of the proton gradient differ from that of genuine amine secreting .. In the pancreatic β-cell the stored insulin ..lex is ..ry sensitive to pH exhibiting maximal ..ity at pH 6.0 (Coore et al 1969), whereas the ..ellular pH has been estimated to be as high as (Hellman et al. 1972). It has also been ..sted that uptake of Ca^{2+} ..nt.. the insulin-con..g β-granules occurs in exchange for protons ..man et al 1979) So far there has been no .. demonstration of .. ΔpH in isolated β-..les or granules from any other polypeptide ..one-producing cell By using a highly sensitive ..metric technique (Salama et al 1980) it has been possible to perform such measurements .. minute amounts of granules that can be ob..d from isolated pancreatic islets.

..out 700 β-cell-rich pancreatic islets were col..ase-isolated from *ob/ob* mice starved over.. A secretory granule fraction was prepared by ..rential centrifugation at pH 7.0 as previously ..ribed (Kohnert et al. 1979). The granule pellet ..suspended in 10 µl 0.25 M sucrose buffered at 7.0 with 10 mM Tris-maleate The suspension ..kept on ice until use. A Aminco-Bowman spectrofluorometer equipped with a 3×3 mm microcuvette was used to measure pH-dependent fluorescence changes of 9-aminoacridine The monochromators were set for narrow-band excitation and emission at 400 and 435 nm respectively Fluorescence changes were recorded on a potentiometric recorder

Each experiment was started by the addition of 90 µl of the Tris-maleate buffered sucrose medium supplemented with 11.1 µM 9-aminoacridine to the cuvette After 1 min, 10 µl of the granule suspension was mixed with the contents of the cuvette The addition of the granules prompted a gradual decrease of the fluorescence signal which reached a steady level within 30 s. (Fig 1). Subsequent mixing with 1 µl 4 M NH_4Cl ga..e a rapid increase of the fluorescence approaching that of 10 µM 9-aminoacridine (the final concentration). The quenching of 9-aminoacridine fluorescence obtained with different granule fractions varied more than could be expected from their protein content. It was checked that the fluorescence was not significantly affected by light scattering from the granules and that the effect of NH_4Cl could not be attributed to a direct interaction with 9-aminoacridine itself

The present technique is based on the ability of amines such as 9-aminoacridine with relatively high pK_a values to permeate freely biological membranes only in their neutral form. The distribution of the protonated form will therefore depend only on ΔpH (Johnson et al. 1978 Salama et al 1980) It is assumed that the fluorescence from 9-aminoacridine taken up by the granules is completely quenched (Salama et al 1980)

In the particular experiment shown in Fig. 1 the addition of β-granules to the 9-aminoacridine medium was associated with .. fluorescence decrease more than 3 fold greater than that expected from dilution alone The kinetics of this decrease was compatible with intragranular accumulation of the amine. After addition of excess NH.. to short

200 mg spheres were suspended in ?0 ml of an equal mixture of Macrodex Tween and distilled water Denser suspensions were difficult to apply without convection The suspension was thermostated in the same water bath When the density gradient was ready the suspension was thoroughly stirred and carefully spread on top of the sucrose solution with the aid of a syringe ?–? 5 h were allowed for sedimentation thereafter the contents of the funnel were withdrawn in 6 fractions

Results The method is somewhat delicate in that small mistakes may cause convection yielding inferior separation In a successful run [illegible] the microspheres were contained in fraction[illegible] standard deviation of diameter of about [illegible] less (Fig ?) The rest of the spheres could [illegible]lected with little loss and rerun.

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...ere hypoglycemia leads to accumulation ...rachidonic acid in brain tissue

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...ow well known that brain tissues only contain ...ll pool of free fatty acid (FFA), of which the ...noic arachidonic and docosahexanoic acids ...rise a minor part (see Bazán 1976, Marion & ... 1979) This pool increases rapidly in ischemia ... nder those circumstances, the polyenoic ...form the major part of the pool Accordingly ... been concluded that ischemia preferentially ...tes phospholipase A_2.

... discovery that arachidonic acid is the sub... of fatty acid cyclooxygenase and thereby the ...rsor of prostaglandins and related substances, ... d to a surge of interest in the FFA metabolism ... ious tissues Thus, since the cyclo-oxygenase ... on is substrate-dependent, accumulation of ...idonic acid may lead to a burst of synthesis of ...aglandins and related substances There is a ...ing suspicion that the cascade of events thus ...ed contributes to reversible and irreversible ... damage (see Marklonis & Garbus 1975 ...er 1979).

...e activation of phospholipase A is not well ... stood but it has been speculated that it occurs ... ditions of cellular energy failure with release ... (Marklonis & Garbus 1975 Flower & ...well 1976). Apart from ischemia, seizures in... by electroshock or pentylenetetrazole ad...tration have been found to elevate brain tissue ...ntration of arachidonic acid (Bazán 1976), ...ervably respiratory distress in animals with ...res could ha e contributed to cerebral energy ...

... this report we give a preliminary account ...ch demonstrating a marked increase in brain ... arachidonic acid concentration during severe ...glycemia, a cond tion leading to ATP depletion ...ut curtailment of tissue oxygen supply

...thod The experiments were performed on ... Wistar rats (230–430 g) that were fasted for ... d then injected with insulin (40 I.U × kg ... at 30 min after the injection the animals were anesthetized with 3% halothane tracheotomized, immobilized with tubocurarine chloride (0.5 mg × kg) and artificially ventilated on about 1% halothane 75% N_2O and 25% O_2. Femoral arterial and venous catheters were inserted for blood pressure recording, sampling of blood, and i.v injections. Electrodes were inserted into the skull bone for EEG recordings, and provision were made for freezing the brain in situ with liquid nitrogen. Ventilation was then continued on 75% N_2O and 25% N_2. Body temperature was maintained at 37°C arterial PCO_2 at 35–40 mmHg and arterial PO_2 at 100 mmHg or higher Uninjected control animals were maintained under similar anesthesia conditions for comparable periods.

Following freezing of the brain in situ and storage at −80°C cortical tissue was dissected at −20°C and extracted with chloroform–methanol After centrifugation and washing the FFA pool of the extract was separated by thin layer chromatography and the free fatty acids were determined with gas-liquid chromatography A full account of the techniques is given elsewhere (Rehncrona et al. 1980)

Results and Disc ssion Following insulin injection blood glucose concentration gradually decreases and when it falls below about 1 μmol × g spontaneous EEG activity ceases (see Agardh et al 1978) In the present series brains were sampled for analyses 5 15 30 and 60 min following cessation of EEG activity

In control animals the FFA concentration of the cerebral cortex was 0 117±0.007 μmol × g (mean ± S.E. n=6). After 5 min of hypoglycemic coma the value increased to 0 751±0 066 μmol × g The FFA pool then remained virtually constant at 15 30 and 60 min of coma. Fig 1 shows values for palmitic (16 0) stearic (18 0) and arachidonic (20 4) acid. Results on the first two of these acids were typical of all others measured xcept one Thus the

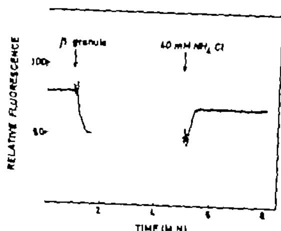


Fig 1 Demonstration of a ΔpH in a β-granule fraction by measurements of the fluorescence quenching of 9-aminoacridine The cuvette initially contained 90 μl 0.5 M sucrose 10 mM Tris-maleate (pH 7 0) and 11 1 μM 9-aminoacridine The fluorescence was quenched by the addition of 10 μl of a β-granule suspension and was enhanced by obliterating the ΔpH by addition of NH_4Cl to a final concentration of 40 mM

circuit the proposed proton gradient there was a rapid release of the amine

When intact pancreatic islets are exposed to 5-hydroxytryptamine or its precursor 5 hydroxytryptophan there is a rapid accumulation of large amounts of the amine within the β-granules (Hellman et al 1972b Gylfe 1978) However as indicated by efflux studies (Gylfe 1978) the amine is not firmly bound within the β-cells Furthermore inhibition of the β-cell metabolism resulted in a rapid depletion of the amine (Gylfe 1980) Since the maintenance of a proton asymmetry in the β-granules would be energy-dependent the latter experimental situation is probably analogous to the addition of NH In summary the present experiments support the existence of a proton gradient in the β-granules which might account for the accumulation of different amines in these organelles

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ibition of fast anterograde axoplasmic transport pressure barrier The effect of pressure dient and maximal pressure

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HAHNENBERGER, R. W Inhibition of fast anterograde axoplasmic transport by pressure barrier The effect of pressure gradient and maximal pressure Acta Physiol Scand 1980 109 117–121 Received 23 March 1979 ISSN 0001–6772. Department of Medical Pharmacology and Ophthalmology University of Uppsala, Sweden.

Fast anterograde axoplasmic transport was studied in the motor fibres of the vagus nerve of rabbits in vitro at 22°C with pressure barrier applied to a small section of the nerve The barrier was characterized by the maximal pressure and the gradients on each side of the maximum. The range tested was 16 to 45 mmHg maximal pressure and 30 to 140 mmHg/mm length of nerve for the pressure gradients. It was found that in the ranges tested the maximal pressure was of major importance for the inhibition of transport, the pressure gradient being of little significance The results indicate that at least one part of fast anterograde axoplasmic transport occurs within collapsible canalicular structures.

Key words rabbit, vagus, axoplasmic transport pressure barrier maximal pressure pressure gradient endoplasmic reticulum, sliding filament

ent years there has been considerable prog- the characterization and understanding of a le role of axoplasmic transport (for a review rafstein 1977). However our knowledge of the nism of this intracellular transport system is agmentary As far as is known different com- ts are transported anterogradely at at least fferent rates one slow and one fast (Grafstein Karlsson & Sjöstrand 1969). This transport ds on local energy supply (Ochs 1971*b*) and inhibited by various agents (Paulsson & Mc 1972, Edstrom & Hansson 1975) of which the itotics are of major importance (Dahlstrom Several mechanisms have been proposed for st axoplasmic transport. Basically there are lternative hypotheses which may be called the ng proces and the canalicul models. worth (1968) first suggested that components migrate along the outer surface of micro- es This idea was developed in more detail chs (1971) He introduced the hypothesis that ling filament loaded with material is trans- d along the microtubules Fast axonal trans- should thus depend on the presence of intact otubules The disruption of their structure by antimitotics (Shelanski 1972) which also inhibit axoplasmic transport (Dahlström 1968) is thus consistent with the sliding filament" hypothesis The canaliculi model on the other hand postulates that an axoplasmic movement takes place within canalicular structures Palay (1958) first pointed out, that the channels of the endoplasmic reticulum might be a possible pathway These structures, like the microtubules, run longitudinally along the axon from the perikaryon to the synaptic region (Droz et al. 1975) Tsukita & Ishikawa 1976). Support for this view was later provided by radioautographic studies by Droz and his group (Droz 1975 Droz et al 1975) and others (Schonbach et al 1971)

In a recent paper a method was described by which it is possible to compress a section of a nerve in vitro with a well defined, adjustable and stable dynamic pressure barrier without interfering with the supply of nutrients and oxygen (Hahnenberger 1978)

The purpose of the present study was to attempt to assess the two models by a physiological approach, based on the following idea. If material moves along a nerve by any mechanism the move

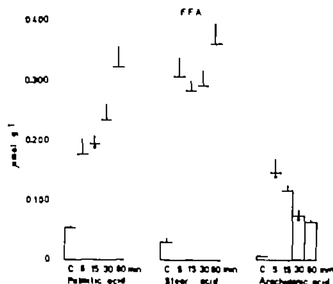


Fig 1 erebral cortical concentrations of palmitic acid (16 0) stearic acid (18 0) and arachidonic acid (20 4) after 5 15 30 and 60 min of isoelectric EEG The values are given in μmol × g wet weight and represent means ± S E. $P<0.05$ $P<0.01$ $P<0.001$

values either increased gradually in the period 5–60 min or remained constant following the increase at 5 min In contrast following a 20-fold rise at 5 min the arachidonic concentration then gradually fell and at 60 min the value was 10 times greater than control

The results thus demonstrate that severe hypoglycemia leads to an increase in the FFA pool similar to that observed in ischemia It is of interest that hypoglycemia neither causes a decrease in cerebral oxygen availability nor a reduction of cellular redox systems (for literature see Agardh et al 1978) However like ischemia it leads to extensive deterioration of cerebral energy state with a reduction in ATP and increases in ADP and AMP concentrations Thus the results corroborate the conclusion that activation of phospholipases occurs when ATP depletion is at hand (Markelonis & Garbus 1975)

Recent results showed that in experiments on renal papillae incubated in vitro the release of prostaglandins was inversely proportional to the glucose concentration of the buffer (Tannenbauer et al 1979) The authors concluded that the formation of prostaglandin was due to release of arachidonic acid from lipid pools in the glucose-deprived tissue

By analogy we tentatively conclude th glycemia a condition leading to accumul arachidonic acid without a reduction oxygen supply should be accompanied by synthesis of prostaglandins and related sub Possibly the gradual reduction of arachid concentration during the course of hyp coma reflects such a synthesis

The skilful technical assistance of Kerstin ... Lena Sjöberg is gratefully acknowledged. The ... supported by grants from the Swedish Medical ... Council (No 14X 263) from U.S. Public Heal... (No RO1 NS07838) and Thorsten and Elsa ... foundation

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Combinations of flow rate in ml/ml and gap size in mm used to produce different maximal [illegible] (P_{max}) and maximal pressure gradient (G_{max})

[illegible]own as gap size/flow rate

	P_{max}, mmHg					
[illegible]Hg/mm	16	22.5	30	32	42	45
	0.4/380		0.4/505			
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LTS

[illegible]1 shows the P_{max} and G_{max} for different set [illegible] flow rate and gap size. P_{max} values up to only [illegible]Hg were used. Above this range any P_{max} [illegible]ccompanied by a G_{max} which could not be [illegible]l to any large extent, even with different [illegible]nations of flow rate and gap size. For exam-[illegible] of 90 mmHg could only be achieved with [illegible]nts ranging between 280 and 320 mmHg/mm. However, it was the aim of the present [illegible] to apply a pressure barrier with a constant [illegible]nd widely different G_{max} or vice versa. Even [illegible]gh pressures could only be obtained at higher [illegible]ents, so there is a correlation between P_{max} and [illegible]

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$$[illegible] \; a_1 P_{max} \; a_2 G_{max}$$

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[illegible] result of the analyses were:

	All points utilized	Two outliers excluded
b_0	−0.55	−0.27
a_1	+0.18; t=1.92*	+0.014 t=5.22***
a_2	+0.0037 t=1.32	+0.0004 t=0.52
F ratio	8.09*	27.5**
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$^*P<0.05$ $^{**}P<0.005$ $^{***}P<0.001$.

The figures showed clearly that in the region of P_{max} and G_{max} tested, P_{max} is the dominant predictor variable for AR, while G_{max} is of insignificant importance—a_1 but not a_2 differs significantly from zero.

DISCUSSION

In previous studies conducted on the same system and run at 22°C axoplasmic flow as partially ar[illegible]rested by a pressure barrier applied to a section of the vagus nerve (Hahnenberger 1978). The present study indicates that in the low pressure range studied (16–45 mmHg) maximal pressure is of crucial importance for the limited transport block observed, the pressure gradient being of little significance. This suggests that the transport that is blocked in the pressure range studied was blocked because structures were collapsed.

These structures might well be the endoplasmic reticulum. In recent years there has been a number of studies in which transported material was localized within the canaliculi of the endoplasmic reticulum. Horse radish peroxidase and acid phosphatase are substances which are transported bidirectionally and are detectable in the endoplasmic reticulum both during anterograde and retrograde transport (Sotelo & Riche 1974, Holtzmann et al. 1955). In the experiments by Droz (Droz et al 1975) on preganglionic axons of the ciliary ganglion

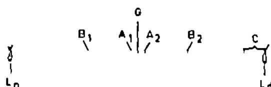


Fig 1 Calculation of the accumulation ratio (AR) A nerve segment between a proximal ligature (L_p) and a distal one (L_d) was subjected to a pressure barrier The peak of the pressure barrier was situated at the centre of the cap (G) Radioactivity detected in the nerve pieces (2 mm each) immediately adjacent to G (A_1 A_2) 8–10 mm proximal and distal to G (B_1 B_2) and this accumulated in the 4 mm nerve piece (C) immediately proximal to L_d was used to form AR For further explanation see text.

ment will be opposed by an existing uphill pressure gradient If this gradient is large enough transport might be blocked On the other hand if transport occurs within canalicular structures this will be impeded by "squeezing" the transporting channels until the lumen collapses This squeezing pressure is defined as the maximal pressure applied to the nerve

MATERIALS AND METHODS

Animals and anesthesia

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Local compression of the vagus nerve

The method including the superfusion fluid is described in detail in a previous paper (Hahnenberger 1978) Briefly a constant flow of fluid was driven through an adjustable gap and directed radially on to the whole circumference of a small section of the nerve The pressure barrier thus applied had a bell shaped profile which was characterized by its maximal pressure P_{max} (maximal height of the curve) and the maximal pressure gradient G_{max} (expressed as the steepest slope of the limbs) By adjusting flow and gap size it was possible to obtain different pressures and different gradients constant over an extended period of time Flow rates and gap sizes providing a particular P_{max} and G_{max} were derived from the calibration curves given in a previous paper (Hahnenberger 1978) rather than determined for each new set up The nerves were superfused at room temperature (22°C) and then cut by a razor blade through the gap. The two nerve segments ... divided in turn into two mm pieces which were ... at least 24 h in ice cold 10% trichloroacetic acid and then analysed for radioactivity as described ...

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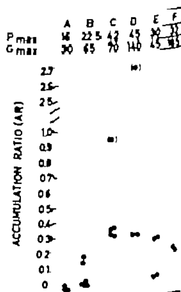


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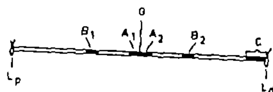


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Statistics

The accumulation ratio (AR) may have been affected by both P_{max} the maximal pressure and G_{max} the pressure gradient Multiple linear regression was therefore calculated using a HP 9810 A desk calculator program STAT PAC IV-1 Significance was calculated with the *t* test according to the formulas of Snedecor (1956)

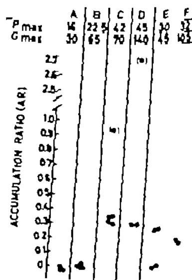


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in the chicken it was found that fast moving labelled proteins are associated with endoplasmic reticulum When the nerve was slightly compressed the lumen of the endoplasmic reticulum dilated in front of the compression giving a high grain density Weiss (1973) has suggested that microtubules might be transporting channels However it seems reasonable that channels such as endoplasmic reticulum consisting of a considerable lumen surrounded by a relatively thin wall should be easier to collapse under the present experimental conditions than microtubules with a small lumen and a comparatively thick wall But it cannot be excluded that microtubules collapse too

Although canalicular transport may exist little is known about the driving force and the mechanism for the intraluminal convection Probably not only microtubules but also contractile proteins play a role (Bray 1977 Roisen et al 1978)

With increasing P_{max} the inhibition of axoplasmic flow was enhanced This might be due to more pronounced changes in all axons and/or to an increase in the proportion of affected axons This problem cannot be solved by the present methods

An important question is if the applied pressure can collapse the axon itself In this case the results would give little insight into intracellular transport systems It can however reasonably be assumed that endoplasmic reticulum is easier to collapse than the whole axon In addition collapsing the axon itself (as in a ligature) would probably destroy the integrity of the longitudinally organized axoplasm and its constituents most likely resulting in irreversible damage to the axon Axoplasmic transport block by this mechanism should thus be reversible But it was found that axoplasmic transport did indeed recover when a pressure barrier of 60 mmHg was applied for only four hours (Hahnenberger 1978) It can thus be assumed that the results presented here mainly reflect intracellular processes

Axoplasmic transport moves insracellular components of widely different sizes from soluble proteins to organelles such as vesicles adrenergic granules and mitochondria Since such organelles have never been described inside the endoplasmic reticulum canalicular transport is probably confined to small sized material perhaps only to the soluble fraction Other transport systems than canalicular convection may thus exist responsible for transfer of material of greater size Bárány (1978) found that at 37°C retrogradely transported adrenergic granules in postganglionic fibres [illegible] able to surmount a pressure gradient of [illegible] mmHg/mm length but not of 30 mmHg/mm [illegible] This suggests that under his conditions [illegible] matter may indeed travel by a sliding process

The present experiments differ from [illegible] Bárány by dealing with anterograde [illegible] 22°C in preganglionic fibres and not [illegible] sively to particulate matter (adrenergic [illegible] Thus the two results cannot be directly [illegible] Moreover the data given here are only valid [illegible] range of the rather low pressures tested [illegible] unfortunately not possible to achieve a [illegible] barrier with every steep gradient but [illegible] pressure At such a barrier (simulating [illegible] gradient might well be the important [illegible] the eye there exists a pressure step between intraocular pressure and tissue pressure of the nerve (Ernest & Potts 1968) This pressure [illegible] established within a very small distance [illegible] less than 0 1 mm A high maximal pressure [illegible] could thus be obtained despite a low [illegible] sure Thus it can still not be decided whether pressure gradient or the pressure step itself [illegible] be suspected of causing glaucomatous [illegible] age

The author thanks Maria Carlsson for excellent [illegible] assistance The investigation was supported by [illegible] 00231-14 from the National Eye Institute US *Health Service to Professor Ernst Bárány*

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WIKBERG and K. L. AXELSSON

ment of Pharmacology Linköping University Sweden

WIKBERG J E S & AXELSSON K L. A simple and efficient method for studying neurotransmitter release in vitro by radiotracer technique. Acta Physiol Scand 1980, 109 123–129 Received 21 Sept 1979 ISSN 0001-6772. Department of Pharmacology Linköping University S eden.

A method which allow measurement of neurotransmitter release from isolated preparations has been developed. With this method release of ^{3}H-acetylcholine from guinea pig ileum and rabbit jejunum could be studied after preincubation of the preparations with ^{3}H-choline The release of noradrenaline from guinea pig vas deferens could also be measured after preincubation with ^{3}H-noradrenaline The method is based on device which allows experiments on 4 preparations at the same time including incubation, field stimulation and collection of fractions for counting of radioactivity With the purpose of obtaining simple and inexpensive device for stimulating the tissue preparations electrically physiological stimulator distributor (PSD) was constructed. It is concluded that the described method constitutes an inexpensive simple and quick means of studying neurotransmitter release from isolated tissues

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METHODS

Rabbits of either sex or male guinea pigs were killed by blow on the head and exsanguinated. In some experiments the vasa deferentia were removed and dissected free from attached mesentery The organs were cut into four 15 mm pieces and mounted on the 4 perspex holders described below In other experiments the guinea pig ileum or rabbit jejunum was dissected as described previously (Wikberg 1977*b*) to obtain innervated specimens of the longitudinal muscle layers. These preparations were also mounted on the perspex holders. The holders were then immersed into scintillation vials containing 8 ml of Krebs solution gassed with 95% O_2 and 5% CO_2 and maintained at tempe rature of 37°C. In order to label the transmitter stores of the adrenergic nerves in the vasa deferentia, these preparations were incubated with 2×10^{-7} M 1-^{3}H-noradrenaline

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simple and efficient method for studying neurotransmitter ease *in vitro* by a radiotracer technique

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A method which allows measurement of neurotransmitter release from isolated preparations has been developed. With this method release of ^{3}H-acetylcholine from guinea pig ileum and rabbit jejunum could be studied after preincubation of the preparations with H-choline. The release of noradrenaline from guinea pig vas deferens could also be measured after preincubation with ^{3}H-noradrenaline. The method is based on device which allows experiments on 4 preparations at the same time including incubation, field stimulation and collection of fractions for counting of radioactivity With the purpose of obtaining a simple and inexpensive device for stimulating the tissue preparations electrically physiological stimulator distributor (PSD) was constructed It is concluded that the described method constitutes an inexpensive simple and quick means of studying neurotransmitter release from isolated tissues.

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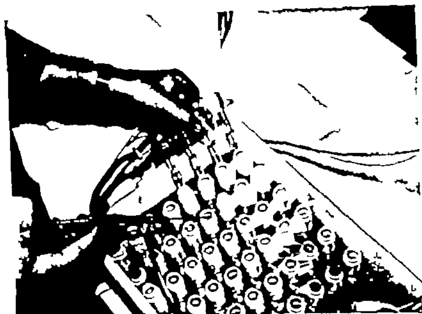
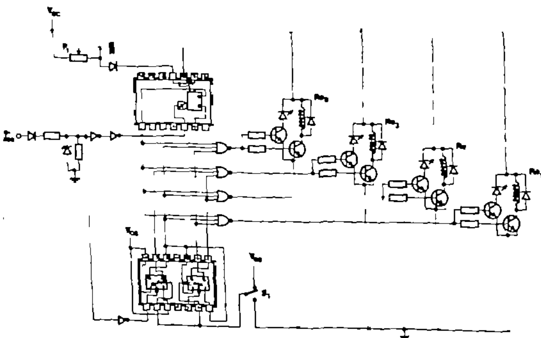


Fig 2 Photograph of the experimental set up. Incubation is performed by immersing the perspex holders into scintillation vials contained in thermostated water bath The vials are mounted at their bottom to stainless steel holders. The incubation medium is delivered by three way automatic syringe



3 A schematic diagram of the physiological stimulator distributor V_{cc} = supply voltage (+5V). P = trim potentiometer Re = relay 0–3 Prepulse indicates trigger input from the Grass stimulator S_1 = reset switch.

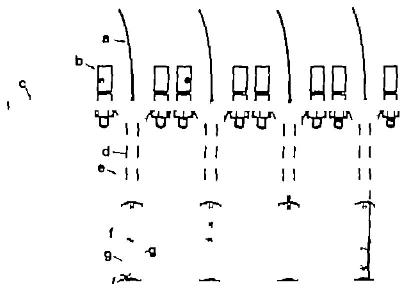


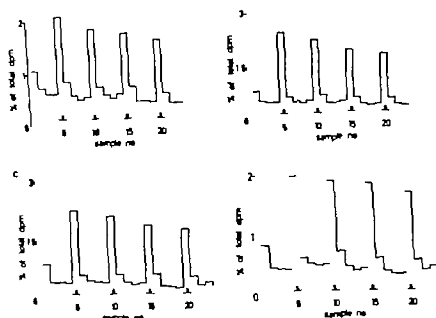
Fig 1 Schematic drawing of the perspex holders (*a*) Polyethylene tubing for oxygen inlet (*b*) Electrical terminal for connection to the PSD (*c*) Perspex board (*d*) Perspex tube (*e*) Insulated platinum wire (*f*) Insulated bars to which the preparation is tied (*g*) Platinum electrodes

(New England Nuclear 60 1 Ci/mol) and ×10 M ascorbic acid for 1 h. To label the transmitter stores of the cholinergic nerves in guinea pig ileum and rabbit jejunum these preparations were incubated with 1×10^{-4} M H-choline (Amersham 13.2 Ci/mol) for 1 h. After incubation the vasa deferentia preparations were washed with Krebs solution containing $2\times10^{-}$ M desmethylimipramine and 1×10^{-4} M normethanephrine The intestinal preparations were washed with Krebs solution containing 1×10 M hemicholinium Electrical stimulation was performed with a Grass SD9 stimulator and a physiological stimulator distributor (PSD) described below The stimulating current was adjusted to 150 mA Higher currents than 300 mA resulted in irreversible damages to the tissues. At the end of the experiments the preparations were dissolved in 0 5 ml soluene (Packard) and the radioactivity was counted in a toluene based scintillator (counting efficiency 40–45%) in a Packard Tri Carb 3375 liquid scintillation spectrometer The radioactivity in the washing medium was counted after the addition of 10 ml of Instagel (Packard) with a counting efficiency of 18–20% The counting efficiencies were determined by use of an internal tandard

Perspex holders The organs were mounted on the 4 perspex holders shown in the schematic drawing in Fig. 1 These holders are constructed from four 75-mm long perspex tubes with an inner diameter of 6 mm Two 15-mm long platinum electrodes are attached at the distal end The preparation is mounted in between these electrodes by tying them to the (insulated) bars situated above and below the electrodes. The 4 holders are mounted at their proximal ends to a perspex board at which electrical terminals for cables from the PSD can be connected The platinum electrodes are electrically connected to these terminals by insulated platinum wires The incubation media can be oxygenated through polyethylene tubings mounted on each holder

For incubating and washing of the preparation holders are immersed (manually) in scintillation vials containing the appropriate medium For this purpose are mounted onto a stainless steel holder contained thermostated water bath (Fig.) These holders were originally used in an agitating machine chased from Grants Instrument England For collection of radioactivity released from the preparations the specimens are washed by manually dipping the holders into new vials The washing periods used in present experiments were min when the guinea pig deferens was studied and 5 min in the expts. on the testinal preparations

Physiological stimulator distributor (PSD). The effect of the SD9 stimulator was too low to allow preparations to be stimulated at the same time We therefore constructed a simple and inexpensive device to this problem Thus in the PSD the signal from the stimulator is multiplexed to 4 output ports by appropriately timed reed relays. The timing of the relays is achieved by the TTL circuitry shown in Fig. 3 The prepulse of the SD9 is fed to a divide by-four counter constructed from two JK flip-flop circuits and a monostable multivibrator (see TTL data book) By decoding the output of these circuits in 4 three-input NAND gates one of the relays can be switched on before the SD9 stimulus pulse is emitted After the emission of the pulse of organ the relay is turned off when the multivibrator reset On the next prepulse this scheme is repeated now the next relay is activated This process continues a circle as long as the SD9 is emitting pulses The device also contains 4 light emitting diodes which are activated when the reed relays are turned on which allows a convenient check that the device is functioning properly copy of the PC-board and parts list of the PSD can be obtained on request)



6 Spontaneous and stimulation evoked release of tritium from four guinea pig vas deferens preparations preincubated with ^{3}H-noradrenaline S = stimulation with 400 volleys at 4 Hz.

in an identical manner as above Electrical field stimulation at 1 Hz for 5 min with 1 ms volleys caused an increase in the release of tritium (Fig. 5). However the stimulation-evoked release was less pronounced than that in guinea pig ileum. To test that the preparation responded to pharmacological agents the effect of noradrenaline was examined, since it has been previously shown that this drug inhibits the release of acetylcholine from the rabbit small intestine as estimated by bioassay (Vizi & Knoll 1971). Noradrenaline (1×10^{-6} M) completely abolished the stimulation-evoked release of tritium (Fig. 5b).

deferens

After incubation of the preparations with ^{3}H-noradrenaline they were washed at 2-min intervals for 60 min. Electrical field stimulation was then performed at 10-min intervals with 400 volleys at 4 Hz with 1.5 ms pulses. The fractions collected during the first stimulation period were discarded, as the release during this period was much larger than during the following ones. After this initial stimulation, fractions were collected for analysis. The electrical stimulation gave rise to a large and consistent increase in the release of tritium. The release was comparable in magnitude in all 4 preparations studied simultaneously (Fig. 6) To test that the preparations responded pharmacologically the effect of 8×10^{-6} M phentolamine was examined It is well known that this drug potentiates transmitter release on nerve stimulation (for references see Starke 1977) The α-blocking agent induced an approximately 3-fold potentiation of the tritium release on field stimulation (data not given) On no occasion was any spurious release of tritium observed.

DISCUSSION

In this paper we have described a method by which the release of noradrenaline or acetylcholine from isolated tissues can be studied as release of labelled transmitter The cost of the equipment was only a fraction of that of conventional experimental set up with the superfusion technique Furthermore the present method allows multiple preparations to be studied simultaneously The handling of the samples is also reduced to a minimum, as incubation and counting of radioactivity are performed in the same vessels. Since a larger amount of experimental data can thus be obtained in a shorter time

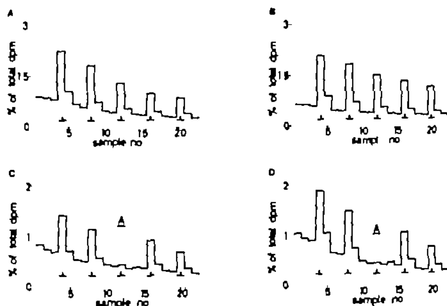


Fig 4 Spontaneous and stimulation evoked release of tritium from four guinea pig ileum preparations preincubated with H-choline S = stimulation with 150 volleys at 0.5 Hz. (A–B) Control (C–D) Addition of 2×10^{-4} M adenosine (A) as indicated

Drugs The following were used. Adenosine (Boehringer Mannheim) hemicholinium bromide (EGA Chemie) and noradrenaline bitartrate (Sigma)

RESULTS

Guinea pig ileum

After incubation of the guinea pig ileum preparations with ³H-choline they were washed 6 times at 5-min intervals for 30 min by incubation in scintillation vials This initial washing was undertaken with the purpose of washing out extracellularly located metabolites (Wikberg 1977*a*) After this time fractions were collected for 5 min each for analysis Electrical field stimulation with 150 pulses at 0 5 Hz and a duration of 1 ms induced a consistent increase in the release of tritium (Fig 4) The tritium release from the 4 preparations studied in the same experiment was comparable in magnitude and on no occasion was any spurious release of tritium observed In order to check that response to pharmacological agents occurred with use of the present method two of the preparations were treated with 2×10 M adenosine since this drug has been reported to inhibit the release of acetylcholine from guinea pig ileum as studied by bioassay (Vizi & Knoll 1976) or by gas chromatography mass spectrometry (Gustafsson et al 1979) The stimulation-evoked release of tritium was completely abolished by the addition of adenosine (Fig 4c–d)

Rabbit jejunum

After incubation of the rabbit jejunum preparations with H-choline they were washed 6 times at 5 min intervals for 30 min as described above for guinea pig ileum Fractions were then collected for 20

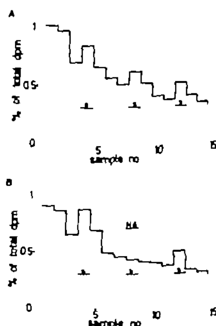


Fig 5 Spontaneous and stimulation evoked release of tritium from two rabbit jejunum preparations preincubated with H-choline S = stimulation with 300 volleys at 1 Hz (A) Control (B) Treatment with 1×10^{-4} M noradrenaline (NA) as indicated

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as compared with the conventional techniques the cost of the experiments is reduced We have chosen to construct a device which allows 4 preparations to be studied simultaneously and we have found that it functions satisfactorily The equipment is very simple to handle However should its capacity be insufficient there is nothing to prevent a similar apparatus being set up for the study of more preparations for example 8 The only disadvantage that could be present with the method is that measurement of mechanical tension of the muscle preparation cannot be performed Due to the complexity of a device which also allowed tension studies we did not implement this facility

We also developed a device to allow simultaneous stimulation of the preparations Naturally several Grass stimulators could be used to achieve the same results but such equipment is rather expensive and it was our aim to develop an inexpensive method The physiological stimulator distributor cost less than 1/10 of one physiological stimulator

In our experiments guinea pig ileum released an increased amount of tritium on electrical stimulation It has been shown that in the presence of eserine the stimulation-evoked release of tritium is almost exclusively attributable to ³H-acetylcholine (Wikberg 1977*a*) We did not use eserine and the released acetylcholine was therefore probably hydrolyzed to ³H-choline In order to be assured that all of the released tritium was collected hemicholinium was added to the incubation medium to block neural reuptake of ³H-choline The stimulation-evoked release of tritium would thus seem valid as a measure of stimulation-evoked release of ³H-acetylcholine Theoretically there may be some problems involved in the use of the radiotracer technique as it may not be certain that the stimulation-evoked release of tritium correlates to the release of endogenous acetylcholine However it has recently been shown that the release of ³H-acetylcholine observed with the radiotracer method correlated well to the release of endogenous acetylcholine measured by gas chromatographic mass spectrometry (Wikberg et al in preparation) Regarding the present technique it should be pointed out that some difficulties might be encountered in studies of the spontaneous release of tritium For instance it could be rather difficult to predict the amount of ³H acetylcholine released into the washing medium The reason for this is that other metabolites such as ³H-phosphorylcholine and ³H choline may be released from radioactive p[illegible] which are probably partly contained in the [illegible] muscle of the preparations (Szerb 1975 Wi[illegible] 1977*a*) Thus in studies of the spontaneous re[illegible] of ³H-acetylcholine the metabolites should be [illegible]rated by some chemical method

Our experiments have also shown that the [illegible] tracer method for measuring the release of a[illegible]choline is applicable to the rabbit jejunum [illegible] results obtained with noradrenaline indicate [illegible] stimulation of adrenergic receptors causes [illegible]tion of acetylcholine release from the [illegible] cholinergic neurons of this preparation This f[illegible] is compatible with previous results obtained [illegible] studies on mechanical tension in rabbit jeju[illegible] which indicated that α receptor stimulation [illegible]ited acetylcholine release (Wikberg 1977*b*)

The method described was also found suitable [illegible] studying the release of ³H noradrenaline from [illegible] vas deferens The results of these experim[illegible] compare favourably to those obtained by sev[illegible] different authors with use of the superfu[illegible] technique (Hedqvist 1974 Wikberg & Ander[illegible] 1978) It is concluded therefore that the pre[illegible] approach can be used as an equivalent or supe[illegible] alternative to previous methods

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ect of sensitization and non antibody IgE on 48/80 luced histamine release from isolated mast cells

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SYDBOM A., KARLSSON T & UVNÄS B Effect of sensitization and non-antibody IgE on 48/80 induced histamine release from isolated rat mast cells. Acta Physiol Scand 1980 109 131 136. Received 27 Sept. 1979 ISSN 0001-6772. Department of Pharmacology Karolinska Institutet, Stockholm, S eden and Department of Medical and Physiological Chemistry Biomedical Centre Uppsala, S eden.

Histamine release from isolated rat mast cells from non-immunized and immunized Hooded Lister rats was induced by compound 48/80 The histamine release was decreased with lower maximum at the optimal concentration of 48/80 when using cells from immunized rats compared to non-immunized control rats. The stimulation of IgE antibody production after immunization using B. pertussis as an adjuvant was also accompanied by an elevation of total serum IgE. The 48/80 induced histamine release from Sprague Dawley mast cell was not inhibited by immunization. Non-antibody IgE showed non-competitive inhibition of 48/80 induced histamine release when myeloma IgE was incubated with mast cells from both Hooded Lister and Sprague Dawley rats. The results indicate the existence of different receptors for IgE and 48/80.

Key ords B pertussis vaccine Hooded Lister rats, immunization, Sprague Dawley rats

phylactic histamine release from mast cells is iated by immunoglobulin (IgE) (Ishizaka & zaka 1966, Johansson & Bennich 1967). The IgE lecule is bound to specific receptors on the mast membranes (Conrad et al 1975). The bridging two IgE molecules on the mast cell surface by igen starts the sequence of events that finally ls to the secretion of histamine and other medi- rs (Levine 1965 Siraganian et al 1975 Segal al 1977)

ompound 48/80, a non-immunological and non- otoxic releaser of histamine (Johnson & Moran 9 Bloom & Chakravarty 1970) has been used for ny years to study the mechanisms of histamine ease from isolated rat mast cells. The histamine ease induced by 48/80 has many features in mmon with that of antigen both morphologically d biochemically (Johnson & Moran 1969 Bloom Chakravarty 1970) However there are some dif ences for example in the influence by e.g Ca^{2+} oreman & Mongar 1972, Uvnäs & Thon 1961) P (Becker & Austen 1966, Ranadive & Cochrane 71 Taylor & Sheldon 1974) or cyclic nucleotides (Johnson et al 1974 Kalmer & Austen 1974 Sullivan et al. 1975 Fredholm et al 1976 Norn et al. 1977) which have raised the question whether there are initially different points of attack for antigen and 48/80 (Uvnäs 1976).

The present investigation was undertaken to study the possible different points of attack for the histamine release mediated by IgE or 48/80 and in particular if changes in the amount of cell bound IgE modifies 48/80 induced histamine release.

MATERIALS AND METHODS

Inbred Hooded Lister rats originating from rats purchased from Møllegaard Avlslaboratorium ApS L. Skensved Denmark, and Sprague Dawley rats from Anticimex, Stockholm, Sweden, were used. Pertussis vaccine (lot. no. 120274) was purchased from Statens Seruminstitut, Copenhagen, Denmark (see Sydbom & Karlsson 1979), Egg albumin from Difco laboratories, Detroit Michigan, USA and human serum albumin (free of preservatives) from Kabi AB Stockholm, Sweden, Ficoll from Pharmacia Fine Chemicals AB Uppsala, Sweden, and compound 48/80 from AB Leo, Helsingborg, Sweden. The IgE was originating from IgE (IR2) secreting tumours in

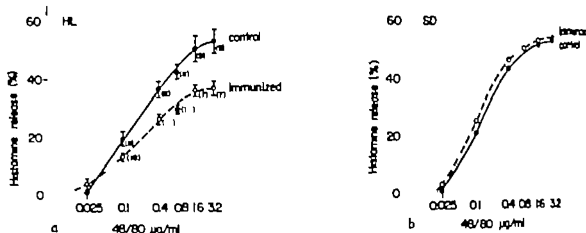


Fig 1 Effect of Immunization on 48/80 induced histamine release from mast cells. Isolated mast cells from n[illegible] munized (●—●) or immunized (O- -O) rats were incubated with compound 48/80 (0 025 to 3 ? µg/ml) at 37°C for [illegible] (*a*) Mast cells from Hooded Lister rats Mean ± S E 1 Number of expts are shown in brackets. With 48/80 conc[illegible] tions above 0 4 µg/ml there is a significant difference between control and immunized mast cells ($p<0\ 01$). Spon[illegible] histamine release was 8 1±0 8% for non-immunized and 9.3±1 0% for immunized rats. (*b*) Mast cells from Sp[illegible] Dawley rats Mean of 2 expts Spontaneous histamine release was 4 9% for non-immunized and 6 1% for im[illegible] rats

LOU/c rats reported by Bazin et al (1973) The monoclonal IgE protein was isolated and purified as described by Bazin et al (1974) All other chemical were obtained from the usual commercial sources

Immunization of rats

Male Sprague Dawley rats (180–?00 g) or Hooded Lister rats (220-3?0 g) were injected s c with ×0 5 ml of B pertussis vaccine together with egg albumin (?0 µg/ml) The animals were killed for isolation of mast cell between day 13 and day ?7 after immunization

Isolation of mast cell

Mast cells from the pleural cavities of Hooded Lister or Sprague Dawley rats were isolated on Ficoll according to the method of Thon & Uvnas (1967) a used earlier (Sydbom & Uvnas 1976)

Incubation procedure

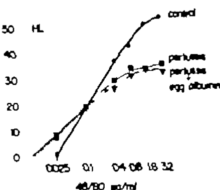
Standard incubation The isolated mast cell were suspended in 2 ml incubation medium 137 mM NaCl 7 mM KCl 1.8 mM $CaCl_2$ 1 mM $MgCl_2$ containing 10% (v/) 67 mM Sörensen s phosphate buffer pH 6 7 and human serum albumin 0 5 mg/ml The mast cell concentration was usually around 5000 cells/ml The mast cell suspension was preincubated at 37°C for 5 min and then 100 µl of a compound 48/80 solution was added to the cell suspension and incubated for another 10 min The reaction was stopped by immersing the tubes in ice-water and centrifuging (350×g) at 4°C for 10 min The supernatants were decanted off into new tubes and 0.. ml 0 1 M HCl was added to the cell residues and the tubes put in boiling water bath for 5 min to extract all histamine 1 8 ml of buffer was added to the cell tubes to readjust the volume Histamine was determined according to the fluorescence technique described by Shore et al (1959) omitting the extraction procedure (Bergendorff & Uvnäs 197?) Histamine release was expressed as % of total histamine cell and supernatant Samples were run in duplicate the spontaneous histamine release has been sub[illegible] from the values presented

Competition between IgE and 48/80 Immunoglob[illegible] (IR2) from a concentrated stock solution (40 mg/ml) incubated with normal Hooded Lister mast cells (a[illegible] 380000 cell) in 100 µl at 37°C for 30 min during or[illegible] ou shaking. 4 tubes were run parallelly with [illegible] only) 0 1 1 and 4 mg IgE respectively After the i[illegible] tion 5 4 ml buffer was added and the cell suspe[illegible] distributed among 10 new tubes each and centrifuged [illegible] min The supernatant were discarded The mast [illegible] were preincubated with ml buffer at 37°C for 5 [illegible] with the addition of 100 µl of 48/80 in 4 different con[illegible] trations (final conc 1 6 0 4 0 1 0 025 µg/ml) for [illegible] 10 min The reactions were stopped and the sample[illegible] dled a described above

RESULTS

48/80 induced histamine release from mast cells from non-immunized and immunized Hooded Lister and Sprague Dawley rats

Isolated mast cells from non immunized H[illegible] Lister rats were incubated in increasing conce[illegible] tions of compound 48/80 at 37°C for 10 min an[illegible] histamine release was determined (Fig 1*a*). Th[illegible] was a maximal histamine release of about [illegible] using mast cells from non immunized rats The dose response curve was significantly sh[illegible] downwards when using mast cells from immu[illegible]



Dose-response curve of 48/80 on histamine release Hooded Lister rat mast cells. Isolated mast cells non-immunized (control) rats (●—●), rats immunized with egg albumin and B pertussis (▼– –▼) or B pertussis alone (■- -■) were incubated with con- 48/80 (0.025 to 3 2 µg/ml) at 37°C for 10 min. Mean expts

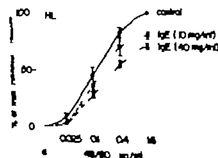
nd the maximum was decreased to about 36% difference in the responsiveness of mast cell non-immunized and immunized Hooded rats could however not be shown with mast from Sprague Dawley rats (Fig. 1*b*). The ase in maximum histamine release can be due overall increase in the IgE production. There- one group of Hooded Lister rats were immun- with B pertussis and egg albumin as before and group of rats were injected with B pertussis as a control. B pertussis is known to be a lator of IgE production in rats (Mota 1958 Tada et al 1972) There was a decrease in histamine release after 48/80 challenge of mast cells from both these immunized groups, as compared to non-immunized control rats but no difference between rats immunized with B pertussis + egg albumin or B pertussis alone (Fig. 2)

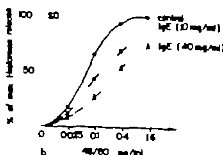
Inhibition of 48/80 induced release from mast cells by non-antibody IgE

Isolated mast cells were incubated in 100 µl with purified non-antibody myeloma IgE (IR2) in 3 different concentrations 1 10 and 40 mg/ml at 37°C for 30 min After dilution and washing the mast cells were incubated with 48/80 0 025 to 1 6 µg/ml Log dose of 48/80 was plotted against % of maximal histamine release High concentrations of non-antibody IgE inhibited the 48/80 induced histamine release from normal Hooded Lister rat mast cells (Fig. 3) There was no effect of the lowest dose of IgE (1 mg/ml) on 48/80 induced histamine release but the dose-response curve was shifted to the right and the maximum was decreased to 80% when the mast cells had been incubated with 10 mg/ml of IgE and to about 70% when 40 mg/ml of IgE was used (Fig. 3) The picture was very much the same when mast cells from Sprague Dawley rats were used (Fig. 3*b*).

DISCUSSION

The original finding of this work was that an over all stimulation of IgE production in Hooded Lister rats by immunization with egg albumin and B per





3 Effect of IgE on 48/80 induced histamine release Isolated mast cells (4 10⁴ cells) were incubated in 100 µl ber with purified myeloma IgE in two different concentrations, 10 mg/ml (■- -■) or 40 mg/ml (▲– –▲) and a rol (●—●) at 37°C for 10 min. After washing and dilution the mast cells were incubated with 48/80 in 4 different entrations at 37°C for 10 min. The histamine release is expressed as percent of maximal release with 1.6 µg/ml 48/80 out IgE (control) and amounted to 44.8±2.2% of total histamine for Hooded Lister rats and to 53.3% for Sprague ley rats. (a) Histamine release from Hooded Lister rat mast cells. Mean ± S E. from 4 expts. (b) Histamine release Sprague Dawley rat mast cells. Mean of 2 expts.

tussis or B pertussis alone inhibited 48/80 induced histamine release from isolated rat mast cells (Fig 1*a*) The maximal histamine release response was decreased about 30% by *immunization* However we could not register any difference in histamine release between non-immunized or immunized Sprague Dawley rats Sprague Dawley rats have very low basic levels of IgE (0–10 ng/ml) compared to Hooded Lister rats (about 600 ng/ml) (Karlsson et al 1979) There are only small variations in the total IgE level after immunization of Sprague Dawley rats (5–10 ng/ml Bennich et al 1978) but the level of total IgE in Hooded Lister rats is increased to about 2 000 ng/ml (Sydbom & Karlsson 1979) This very pronounced difference in IgE between the two rat strains could perhaps explain why the histamine release induced by 48/80 could be decreased by immunization of Hooded Lister rats but not Sprague Dawley rats To investigate whether this inhibition was due to IgE we incubated the mast cells with purified non-antibody IgE from IgE secreting myeloma cells The results showed that there was a non-competitive inhibition of 48/80 induced histamine release with increasing IgE both when mast cells from Hooded Lister rats and Sprague Dawley rats were used However if we calculate the IgE in the incubation medium there is a great excess of IgE The molecular weight of rat IgE is about 190 000 (Bennich et al 1978) and the number of receptors on a mast cell surface around 300 000 (Conrad et al 1975) In our experiments we used 4 mg of myeloma IgE to 380 000 mast cells which will give 1 ?$\times 10^6$ molecules of IgE per receptor With this excess all receptors ought to be occupied and it is rather surprising that there could be a differentiation between the inhibitory effect of the two doses of IgE 10 and 40 mg/ml

If the amount of IgE molecules attached to the mast cell receptors is a *reflection of the serum level* of IgE the immunized Hooded Lister mast cell might be covered by IgE and there would probably be a steric hindrance for 48/80 to react with its receptor and the dose response curves indicated a non-competitive inhibition (Fig 3) The serum level of IgE in immunized Sprague Dawley rats is low and the concentration of IgE molecules on the mast cell surface consequently low This could explain why immunization of Sprague Dawley rats does not inhibit 48/80 induced histamine release (Fig 1*b*) The number of IgE molecules on human basophils correlate well with the serum IgE level (Conroy et al 1977) Sobotka & coworkers (1978) [illegible] the number of basophil receptors also corr[illegible] the serum IgE levels. However mast c[illegible] *non-immunized Sprague Dawley* rats hav[illegible] free IgE receptors than Hooded Lister [illegible] there is no difference in the total uptake of [illegible] tween mast cells from Hooded Lister and Sp[illegible] Dawley rats (Karlsson 1978) indicating [illegible] total number of IgE receptors are the same [illegible] two rat strains

Hino et al (1977) have reported evide[illegible] specific binding of 48/80 to mast cell surfa[illegible] would indicate the existence of a 48/80 re[illegible] However Morrison et al (1975) have ma[illegible] minary experiments that would suggest 48[illegible] IgE to act on the same receptor This co[illegible] was drawn out of desensitization studies [illegible] cells from immunized rats and desensitized [illegible] gen did not respond to 48/80 In view of our [illegible] this could perhaps also be explained by [illegible] hindrance by IgE In conclusion our find[illegible] a stimulation of IgE production in Hooded [illegible] rats inhibits 48/80 induced histamine release [illegible] fact that non-antibody IgE non-competitive[illegible] hibits 48/80 induced histamine release indica[illegible] existence of different receptors for IgE and [illegible] and different points of initial attacks for the [illegible] lation of secretion

This study was supported by grants from the S[illegible] Medical Research council (B78-04X 39-14B and [illegible] No 13X 3556) and Swedish Society of Medical Sc[illegible]

A preliminary report of these results was pre[illegible] the eighth annual meeting of the Histamine Club [illegible] pean Branch) May 16–18 1979 in Stockholm

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ATP β-γ-methylene-ATP and adenosine inhibit non-cholinergic non-adrenergic transmission in rat urinary bladder

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DAHLÉN S.-E. & HEDQVIST P ATP β-γ-methylene-ATP and adenosine inhibit non-cholinergic non-adrenergic transmission in rat urinary bladder Acta Physiol Scand 1980, 109 137–142. Received 1 Oct. 1979 ISSN 0001-6772. Department of Physiology Karolinska Institutet, Stockholm Sweden.

ATP and adenosine caused dose-dependent and reversible inhibition of the atropine resistant contraction response to transmural nerve stimulation in the rat urinary bladder Both purines also inhibited contraction responses to acetylcholine and direct muscle stimulation, indicating postjunctional effect on the transmission. It seems as ATP per se inhibits the excitatory transmission, because the stable ATP-analogue β-γ-methylene-ATP was inhibitory as well, and because exogenous adenosine deaminase abolished the inhibition by adenosine but not that by ATP or β-γ-methylene ATP Blockade of purine inactivation enhanced the inhibitory action of ATP and adenosine, and by itself inhibited the transmission. These results are consistent with the possibility that endogenous purines may modulate non-cholinergic non-adrenergic excitatory transmission in the rat urinary bladder

Key words ATP adenosine, β-γ-methylene ATP non-cholinergic non-adrenergic transmission, rat urinary bladder blockade of purine inactivation, transmural nerve stimulation

is considerable morphological evidence that mammalian urinary bladder is cholinergically innervated (e g El-Badawi & Schenk 1966). Yet, it is well established that atropine fails to block nerve-induced contractions in the urinary bladder (Langley & Anderson 1896) This phenomenon has commonly been explained in terms of an atypical cholinergic transmission with junctional receptors that are inaccessible to atropine (Dale & Gaddum 1930 Carpenter 1977) However it has also been suggested that part of the excitatory transmission is non-cholinergic (Henderson & Roepke 1934) and, more specifically "purinergic" (Burnstock 1971 Burnstock et al 1972, 1978). ATP undoubtedly contracts the urinary bladder of most species (e g. Buchthal & Kahlson 1944) and ATP is reported to be released by intramural nerve stimulation in the guinea pig urinary bladder (Burnstock et al 1978). Still the hypothesis of purinergic transmission has been challenged mainly on the grounds that ATP-contractions in many respects imperfectly mimic the response to nerve stimulation (Ambache & Zar 1970 Ambache et al. 1977 Ambache 1978).

Purines and in particular adenosine have been suggested as modulators of adrenergic and cholinergic transmission (Hedqvist & Fredholm 1976, Sawynok & Jhamandas 1976 Vizi & Knoll 1976) We report that ATP its degradation product adenosine and the stable ATP-analogue β-γ-methylene-ATP (β-γ-M ATP) inhibited nerve-induced contractions in the isolated rat urinary bladder which raises the possibility that endogenous purines may modulate non-cholinergic non-adrenergic transmission as well

A preliminary account of part of the results was presented at the XVI Scandinavian Congress of Physiology and Pharmacology 1979 (Dahlén 1979).

MATERIAL AND METHODS

Female Sprague-Dawley rats (250 g), were killed by blow on the head and bled. The urinary bladder was

isolated and two mucosa-free strips prepared from the detrusor part. Unstretched the strips were approximately 5×15 mm with a thickness of 1–3 mm The preparation was suspended in a 5 ml organ bath containing Krebs-Henseleit s solution (composition (mM) NaCl 143 KCl 5 6 $CaCl_2$ 2.5 MgCl 1 0 NaHCO 11 9 glucose 5 5) The solution was kept at 37°C and gassed with 5% CO in O_2. Washout was performed by changing the whole bath volume Unless otherwise stated biphasic pulses of 0.5 ms duration and of supramaximal voltage were delivered by a Grass S4 stimulator through platinum wire electrodes along the wall of the organ bath Contractions were recorded on a Grass Model 7 Polygraph via an isometric Grass FT 03C force displacement transducer (resting tension 5–10 mN) In some experiments mechanical activity was registered isotonically (load 0.5 g) with a Harvard heart/smooth-muscle transducer on a Honeywell ink writer As regards the inhibitory action of ATP and adenosine both recording methods gave the same result

Drugs (reagent grade) acetylcholine chloride (ACh Sigma) atropine sulfate (Sigma) tetrodotoxin (Sigma) hexamethonium bromide (May & Baker Ltd) adenosine 5 triphosphate disodium salt (ATP Sigma) adenosine (Sigma) β-γ-methylene adenosine 5 -triphosphate sodium salt (β-γ-M ATP Sigma) dipyridamole (provided by Boehringer Ingelheim AB) guanethidine bisulfate (provided by Hässle-Ciba-Geigy AB) adenosine deaminase (ADA Sigma, type II crude powder Act IU 1 μmol adenosine/min pH=7.5 T=25°C) erythro-9-(2 hydroxy 3-nonyl)-adenine (=EHNA provided by Burroughs-Wellcome) Since atropine had little and guanethidine no effect on the contraction response to transmural nerve stimulation these drugs were not routinely added to the bath fluid

The effect of drugs on the response to transmural nerve stimulation was calculated from the ratio between the average height of 5 contractions immediately preceding drug addition and that of 5 contractions after a stable drug effect was obtained Statistical hypotheses were tested by Student s t test for paired and unpaired variates

RESULTS

Strips of rat detrusor muscle consistently responded to transmural stimulation (1–200 pulses 1–40 Hz) with a prompt contraction This contraction was abolished by tetrodotoxin (0 31 μM) but left unaffected by guanethidine (1–5 μM) or hexamethonium (3–30 μM) After atropine (0 1–1 0 μM) the contraction caused by ACh (0 01–100 μM) was completely blocked while the contraction response to transmural stimulation (10 pulses 1–5 Hz) was at most reduced by 30% (mean ± S E =13 8±3 0% n=11 P<0 001) Thus the contraction obtained by transmural stimulation of rat urinary bladder is nerve-mediated resistant to adrenergic and ganglionic blockade and only moderately reduced by blockade of muscarinic receptors

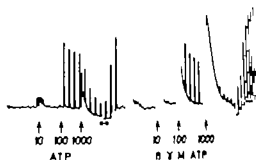


Fig 1 Dual effect of ATP and β-γ-M ATP on rat urinary bladder direct contraction but inhibition of responses to transmural nerve stimulation (5 Hz, 10 pulses) at 1 min intervals. Drugs added at arrows, ... dots

This is in agreement with previous findings in ... species including the rat (e g. Ambache & ... 1970)

ATP (1–15000 μM) initially caused a ... and dose-dependent contraction of the rat de... strip but there was always a marked tachyph... to repetitive administration of one and the ... dose of ATP The contraction response to ... tent transmural nerve stimulation (10 pulses ... at 1 min intervals) was however never ... but rather dose-dependently depressed b... (Fig 1) The inhibitory effect which show... tachyphylaxis was manifest within 30 s, ... reached its maximum within 2–3 min and re... so until the preparation was washed

The stable ATP-analogue β-γ-M ATP (1-... μM) as previously reported (Brown et al. ... caused a stronger initial contraction than ATP ... 1) But as ATP it dose-dependently inhibit... contraction response to transmural nerve st... tion (Fig. 1)

Adenosine (1–1000 μM) relaxed the non-... lated preparation Moreover adenosine do... pendently depressed the response to tran... nerve stimulation (Fig 2 3) and in this re... was more potent than ATP (Fig 3). Aden... (10 μm) inhibited the nerve induced contr... 20.5±1 4% before and 15 2±7 2% after at... 0.5 μM (means ± S E n=15 and 5 respec... P>0.2) It is unlikely therefore that the inh... by adenosine reflects interactions with ... cholinergic part of neuroeffector transmission

To test whether ATP inhibited the contr... response secondarily to being metabolized ... adenosine ADA was ad... o the organ b...

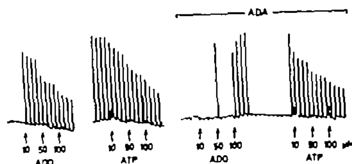


Fig. 2. Isolated rat urinary bladder contraction response to transmural nerve stimulation (5 Hz, 0.5 ms, 10 pulses) at 1 min intervals. Adenosine deaminase (ADA 0.2 units/ml) antagonizes the inhibition by adenosine but not that by ATP.

xperiments. However while ADA effectively nteracted the effect of adenosine (Fig. 2), the ory action of ATP (Fig. 2) and β-γ-M ATP own) was present as before ADA. By itself, had no effect on the response to transmural stimulation.

r blockade of nerve-propagated responses to ural stimulation by tetrodotoxin (0.3 μM) a muscle stimulation was accomplished by of pulses with long duration (5–25 ms). ame (100 μM) inhibited the ensuing contrac the same extent as the nerve-induced con- n (Fig. 4). Furthermore, the contraction d by ACh (1 50 μM) was dose-dependently ted by ATP adenosine and β-γ-M-ATP (Fig. 5). Adenosine was somewhat more potent than the two other substances (Fig. 5).

Adenosine action is known to be terminated both by tissue uptake and by deamination to inosine (Arch & Newsholme 1978). Dipyridamole (0.5–1 μM) has been reported to reduce adenosine uptake into various tissues by 75–95% (Satchell et al. 1972, Dieterle et al. 1978, Moritoki et al. 1978) and EHNA (1 μM) inhibited adenosine deaminase activity by 96% in rabbit renal homogenates (Fredholm et al. 1978). In the present experiments, dipyridamole (1 μM) caused a slight, but significant, inhibition of the contraction response to transmural nerve stimulation (10±3.0% mean ± S.E. $n=10$, $P<0.001$) whereas EHNA (1 μM) was ineffective

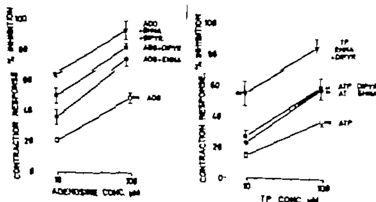


Fig. 3. Isolated rat urinary bladder. Effect of dipyridamole 1 μM, (■), EHNA, 1 μM, (●) and dipyridamole 1 μM, +EHNA, 1 μM (▼) on the inhibition by adenosine and by ATP of contraction of responses to transmural nerve stimulation (5 Hz, 0.5 ms, 10 pulses) at 1 min intervals. Mean values ± S.E. number of expts. within parentheses. EHNA or dipyridamole significantly ($P<0.05$) increased the inhibition. EHNA + dipyridamole caused further increase of the inhibition (significant at least at the 5% level, except when comparison was made with dipyridamole + 100 μM adenosine).

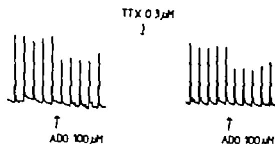


Fig 4 Inhibition by adenosine of contraction responses to transmural nerve stimulation (5 Hz 0.5 ms, 10 pulses) and to direct muscle stimulation (5 Hz 15 ms 10 pulses) after nerve paralysis by tetrodotoxin

in this respect When the two drugs were given together there was no additive effect on the response to electrical stimulation The inhibitory effect of adenosine (10–100 μM) on the contraction response to transmural nerve stimulation was markedly increased by dipyridamole or EHNA (Fig 3) The combination of EHNA and dipyridamole further increased the inhibitory effect of adenosine (Fig 3) Dipyridamole and EHNA also increased the inhibition by ATP (10–100 μM) of contraction responses to transmural nerve stimulation and more so when the two drugs were given together (Fig 3) Noticeably the inhibitory action of β-γ-M ATP) (10–100 μm) on elicited contraction responses was also increased after dipyridamole+EHNA (Fig. 6)

DISCUSSION

Adenosine and ATP dose-dependently inhibited the contraction response to transmural nerve stimulation in the rat (present report) and guinea pig urinary bladder (Dahlén 1979) It has been reported that ATP may increase spontaneous activity and contraction response to intramural nerve stimulation in the guinea pig urinary bladder superfused with a modified Krebs solution containing approximately 0 10 mM magnesium (Burnstock et al 1978) In our experiments however ATP never increased spontaneous activity and the contraction response to transmural stimulation was consistently depressed by ATP even if the nerve-induced contraction was superimposed on an ATP twitch (Figs 1 and 6) Furthermore when using a similar modified Krebs solution as Burnstock et al or even a magnesium free solution we have observed but inhibition both in the rat and guinea pig bladder (Dahlén & Hedqvist unpublished)

Purines inhibit the release of neurotrans[illegible] many autonomically innervated tissues [illegible] Hedqvist & Fredholm 1976) but a similar [illegible] the urinary bladder cannot be evaluated [illegible] exact nature of the transmission is revealed [illegible] ever the present finding that ATP and ade[illegible] depressed the response to both direct muscle [illegible]lation and ACh provides evidence that bo[illegible] and adenosine have a postjunctional [illegible] effect

The observed inhibition by ATP of contr[illegible] responses is somewhat unexpected in the [illegible] ATP as a proposed excitatory transmitter [illegible]tedly the inhibition might be due [illegible] metabolism of ATP forming inhibitory degr[illegible] products such as adenosine (Brown et al [illegible] However the present experiments strongly s[illegible] that ATP by itself is inhibitory Thus, the [illegible] ATP analogue β-γ-M ATP also inhibited the [illegible]traction response to transmural nerve stimu[illegible] In addition treatment of the tissue with ADA [illegible]tively annulled the inhibition by adenosine, [illegible] that by ATP

It has been proposed that there are several [illegible] receptors (Burnstock 1976) and it is evident [illegible] the rat urinary bladder one and the same [illegible] e g ATP may produce both excitation and [illegible]tion thus suggesting the presence of at least [illegible] postjunctional purine-sensitive sites Neverthe[illegible] the main effect of added ATP on nerve-elicited [illegible]traction responses appears to be at the [illegible]

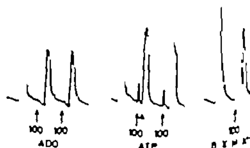
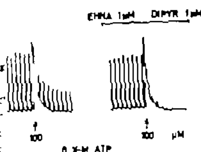


Fig 5 Inhibition by adenosine ATP and β-γ-M [illegible] (added at arrows) of contraction responses to 50 μM [illegible] (A) in isolated rat urinary bladder Preparation [illegible] s after each ACh-administ[illegible]



Isolated rat urinary bladder contraction responses ... ymural nerve stimulation (5 Hz, 0.5 ms, 10 pulses) ... in intervals. Inhibitory effect of β-γ-M-ATP en... l by dipyridamole EHNA. Note that contraction ... ses are inhibited even ben superimposed on the ...contraction by β-γ-M-ATP

which could seem to be at variance with the pt of "purinergic" excitatory transmission in ssue

reasonable to assume that dipyridamole and A in the concentrations used, cause a major tion of adenosine inactivation (e.g. Satchell et 72 Fredholm et al 1978) Accordingly these enhanced the inhibitory effect of adenosine rve-induced contractions Noteworthy also nhibition by ATP was increased after di- amole and/or EHNA, which as suggested by s (Satchell et al 1972, Arch & Newsholme might be due to product inhibition of ATP dation when adenosine accumulates But, it is ely that such mechanisms contribute to the ented inhibition by β-γ-M ATP after di- amole plus EHNA. It is, however possible both ATP per se and β-γ-M-ATP might be vated via e g. the same uptake mechanism as sine

seems well established that stimulation of ad- gic (Su 1975 Fredholm & Hedqvist 1978), nergic (Sulesky 1975) and non-cholinergic adrenergic (Burnstock et al. 1970) nerves es a release of purines of which adenosine is to be a major component (Satchell & stock 1971 Fredholm & Hedqvist 1978) stock et al (1978) reported that intramural stimulation of the superfused guinea pig uri- bladder released ATP but release of other nes was not looked for Endogenous purines, in icular adenosine, have been suggested to func- as modulators of adrenergic and cholinergic roeffector transmission (Hedqvist & Fredholm 1976 Sawynok & Jhamandas 1976 Vizi & Knoll 1976 Gustafsson et al 1978) The present finding that ATP and adenosine inhibited nerve-induced contractions in the rat urinary bladder might indi- cate a modulatory role for purines also in non- cholinergic non-adrenergic transmission. The ob- servation that dipyridamole by itself inhibited the transmission is consistent with this view Since purines could derive both from nervous and non- nervous sources the possibility of endogenous purines modulating the non-cholinergic non-ad- renergic excitatory transmission in the rat urinary bladder is not critically dependent of whether purinergic nerves exist or not.

This study was supported by grants from the Swedish Medical Research Council (project 04X-4342) Karolinska Institutet and Magnus Bergvall Foundation.

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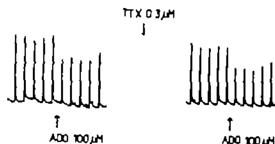


Fig 4 Inhibition by adenosine of contraction responses to transmural nerve stimulation (5 Hz 0.5 ms 10 pulses) and to direct muscle stimulation (5 Hz 15 ms 10 pulses) after nerve paralysis by tetrodotoxin.

in this respect When the two drugs were given together there was no additive effect on the response to electrical stimulation The inhibitory effect of adenosine (10–100 μM) on the contraction response to transmural nerve stimulation was markedly increased by dipyridamole or EHNA (Fig 3) The combination of EHNA and dipyridamole further increased the inhibitory effect of adenosine (Fig. 3) Dipyridamole and EHNA also increased the inhibition by ATP (10–100 μM) of contraction responses to transmural nerve stimulation and more so when the two drugs were given together (Fig 3) Noticeably the inhibitory action of β γ-M ATP) (10–100 μm) on elicited contraction responses was also increased after dipyridamole+EHNA (Fig. 6)

DISCUSSION

Adenosine and ATP dose-dependently inhibited the contraction response to transmural nerve stimulation in the rat (present report) and guinea pig urinary bladder (Dahlén 1979) It has been reported that ATP may increase spontaneous activity and contraction response to intramural nerve stimulation in the guinea pig urinary bladder superfused with a modified Krebs solution containing approximately 0 10 mM magnesium (Burnstock et al 1978) In our experiments however ATP never increased spontaneous activity and the contraction response to transmural stimulation was consistently depressed by ATP even if the nerve-induced contraction was superimposed on an ATP twitch (Figs 1 and 6) Furthermore when using a similar modified Krebs solution as Burnstock et al or even a magnesium free solution we have observed but inhibition both in the rat and guinea pig bladder (Dahlén & Hedqvist unpublished)

Purines inhibit the release of neurotrans... many autonomically innervated tissues Hedqvist & Fredholm 1976) but a similar ... the urinary bladder cannot be evaluated ... exact nature of the transmission is revealed ... ever the present finding that ATP and ade... depressed the response to both direct muscle ... lation and ACh provides evidence that both ... and adenosine have a postjunctional ... effect

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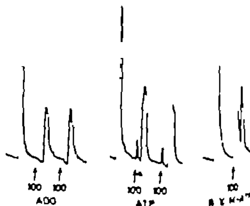


Fig 3 Inhibition by adenosine ATP and β-γ-M ... (added at arrows) of contraction responses to 50 μM ... (▲) in isolated rat urinary bladder Preparation was ... s after each ACh-administration.

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[The] effect of polycythemia on blood flow [in] working and non working skeletal muscle

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GUSTAFSSON L. APPELGREN L. & MYRVOLD H. E. The effect of polycythemia on blood flow in working and non-working skeletal muscle. Acta Physiol Scand 1980 109 143–148. Received 1 Oct 1979 ISSN 0001-6772. Department of Surgery I and II and Department of Anaesthesiology III University of Göteborg, S eden

The effect of acutely induced polycythemia on blood flow and iscosity in the vasodilated vascular bed of working and non-working skeletal muscle was studied In 12 mongrel dogs anaesthetized with thiopental sodium the calf muscle of one hind limb wa isolated Vasodilation was induced either by sciatic stimulation setting the muscle at maximal work or by i.a infusion of papaverine. Blood flow was measured at different perfusion pressures before and after infusion of 300 ml packed homologous red cells. Blood viscosity in vitro was determined in complate viscometer Apparent viscosity in viv was analyzed by comparing pressure-flow relationships for blood and a reference solution. Polycythemia decreased blood flow by 35% in the non-working muscle but less than 10% in the working muscle at comparable perfusion pressures Blood viscosity in vitro increased by 35% at low shear rates Apparent viscosity in vivo increased by 35% in the non-working muscle but less than 10% in the working muscle. The flow impairment induced by polycythemia was far more pronounced in the non-working skeletal muscle indicating a flow facilitation by the rhythmic muscle contractions Erythrocyte flow in fact increased in the working muscle after induced polycythemia while decreased in the non-working muscle.

Key ords Skeletal muscle blood flow exercise polycythemia, viscosity

[Acute]ly induced polycythemia is associated with [a decre]ase in cardiac output and venous return [(Murray]on & Richardson 1961 Replogle & Merrill [1970,] Wense et al. 1964) The change in blood flow [in] polycythemia seems to be approximately re[ciproc]al to changes seen in blood viscosity in vitro. [The] higher oxygen carrying capacity offered by [polyc]ythemia is therefore to some degree out[weigh]ed by the decrease in flow due to increased [viscos]ity

[In s]keletal muscle the blood flow is dependent on [hema]tocrit and there is a decrease of flow at in[creas]ing hematocrits (Levy & Share 1953 Messmer [et al.] 1972, Whittaker & Winton 1933). The appar[ent v]iscosity in vivo is however considerably lower [than] that in vitro (Djojosugito et al. 1970 Levy & [Shar]e 1953 Whittaker & Winton 1933) Fur[therm]ore the effect of hematocrit on apparent viscosity in vivo is less pronounced than on viscosity in vitro (Levy & Share 1953 Whittaker & Winton 1933) This can be explained by the Fåhraeus-Lindqvist effect acting in small vessels and reducing viscosity in vivo (Djojosugito et al 1970)

These conclusions are based on experiments performed during resting conditions. During physical performance a small increase of hematocrit might, however be of benefit for the capacity of the organism (Ekblom et al. 1972 1976) During such conditions the advantage of increased oxygen carrying capacity seems to be more important than the disadvantage of increased blood viscosity

It was the purpose of this study to investigate the effect of polycythemia upon pressure-flow relationships and on apparent viscosity in vivo in the isolated working and non-working calf muscle of the dog.

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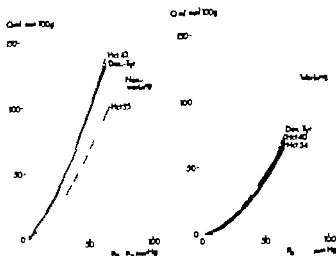


Fig. 3. Pressure flow relationships for Dextran-Tyrode solution (—) in the working and vasodilated non-working muscle in relation to the pressure-flow relationships for blood as taken from Fig. 2. For clearness spread of data was omitted.

tal volume of red cells transported to the muscles ime as an indication of oxygen availability was d at normal hematocrit and after polycythemia by ng hematocrit by the flow rate (Guyton & on 1961).

ional values for apparent blood viscosity in vivo ent perfusion pressures were calculated from the -flow curves for blood and for oxygenated Dex rode solution, Newtonian solution with con- cosity of 2.2 cp assuming the blood and reference viscosities in the calf muscle to be inversely onal to the registered flows at certain perfusion s.

sity in vitro of whole blood and plasma were de- d in Wells-Brookfield synchroelectric cone-plate eter model LVT using 1 ml samples of hep- l blood and plasma. Viscosity measurements ade on two samples at each hematocrit and blood ken simultaneously with the flow measurements. urements were made at shear rates of 23, 46, 115 and 230 s^{-1}. Hematocrits were measured in micro-hematocrit tubes.

For statistical analysis the Wilcoxon matched-pairs signed-rank test was used.

RESULTS

After transfusion hematocrit increased in all dogs. Hematocrit increased from 42±1.2 to 55±0.5 (mean ± S.E.) in the group with non-working muscle and from 40±1.1 to 54±1.[illegible] in the group with working muscle. After acutely induced polycythemia there was a numeric decrease of flow in both non-working and working skeletal muscle. In the non-working group, the flow was 138±6 ml·min⁻¹ 100 g⁻¹ (mean ± S.E.) at a perfusion pressure of 70 mmHg with normal hematocrit and 100±5.5 ml·min⁻¹ 100 g⁻¹

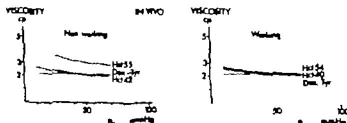


Fig. 4. Average relationship between perfusion pressure and calculated apparent viscosity in vivo in the working (—) and vaso-dilated non-working ( ) skeletal muscle at normal hematocrit and after induced polycythemia.

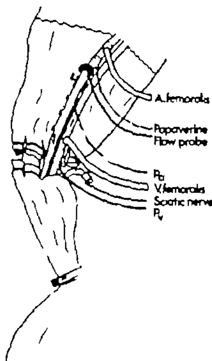


Fig 1 The isolated calf muscle of the dog.

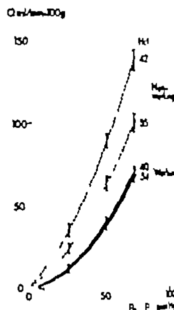


Fig 2 Pressure flow relationships in the work[illegible] and vasodilated non working ( ) skeletal [illegible] normal hematocrit and after induced [illegible] Spread of data is given in 3 representative [illegible] sion pressures of 25 50 and 70 mmHg (mean [illegible]

MATERIALS AND METHODS

[illegible] mongrel dogs (body weight 15– [illegible] kg) were anesthetized with i v thiopental sodium (15 mg/kg) with repeated doses (2–4 mg/kg) throughout the expt The dogs were intubated endothraceally and ventilated with air using a volume controlled respirator Blood gases were controlled and adjusted throughout the expt

The flow measurements were performed on the isolated calf muscle of one hind limb of the dog The experimental model was in principle that described in previous studies (Djojosugito et al 1970 Gustafsson et al 1977) but with some modifications (Fig 1)

The thigh muscles were divided above the knee joint, as were all vessels except the popliteal artery and vein The skin was loosened around the calf in order to exclude cutaneous blood flow after which the skin was re positioned thus protecting the preparation The paw was excluded by a screw clamp just above the ankle joint The femoral superficial artery was isolated and all side branches were divided except two which were used for pressure registration and drug infusion

Wide-bore siliconized T-tubes were introduced in the femoral artery and vein for intermittent extracorporeal perfusion of the isolated limb with oxygenated Dextran-Tyrode solution (6% Dextran 40 in Ringer s solution) from a pressure bottle connected to the arterial T-tube

The perfusion pressure was measured with Statham pressure transducers, model P 23 [illegible] arterial and vein catheters and was registrated continuously on a Grass polygraph model 7 C In order to obtain different perfusion pressures an adjustable screw clamp was placed on the artery proximal to the pressure catheter Blood flow was measured with an electromagnetic flow meter [illegible] (model 37[illegible]) with the flow probe placed on the [illegible] proximal to the screw clamp Another side branch artery was used for infusion of papaverine

Polycythemia was induced by transfusion of [illegible] ml packed homologous red cells into a central [illegible] cells are allowed to mix in the animal for about [illegible] before new measurements were performed

In six dogs flow measurements were performed [illegible] muscles set at heavy rhythmical exercise by [illegible] the cut end of the sciatic nerve with square wave [illegible] from a Grass stimulator The stimulation character[illegible] were 3–5 V 3–5 ms and 4–6 impulses per second [illegible] flow measurements were performed on the rest[illegible] cles where the vascular bed was vasodilated by [illegible] ous i a infusion of papaverine (80 mg/min)

Pressure-flow curves were constructed by step[illegible] justments of the screw clamp The pressure-flow [illegible] hips in the working and vasodilated non-[illegible] muscles were established at normal hematocrit and polycythemia for perfusion pressures between [illegible] mmHg By adjusting the screw clamp in distinct [illegible] blood flow expressed in ml per minute per 100 [illegible] obtained as a function of perfusion pressure [illegible] hematocrit the screw clamp was adjusted from [illegible] flow down to complete closure and reopened [illegible] about 20 distinct steps altogether maintaining the [illegible] sion pressure constant for 5–10 s at each step The [illegible]ing-reopening procedure was repeated three to four [illegible] and the values of flow and perfusion pressure [illegible] were plotted on a pressure flow diagram. Pressure [illegible] curves were also registrated for the oxygenated D[illegible] Tyrode solution of const

-obtained by papaverine compared to metabol- induced vasodilation as demonstrated by gens et al (1975) who during infusion of ade in the exercising muscle saw an increase of flow by approximately 25–30% compared to flow during exercise alone.

apparent viscosity in vivo could be calcu- by perfusing the limb with a reference solution own viscosity (Djojosugito et al. 1970). Since an-Tyrode solution behaves as a Newtonian the pressure-flow curve for this perfusate will physical description of the vascular bed, i.e. bration curve for the biological viscometer comparison of flow values for blood and Dex yrode solution at identical perfusion pres- apparent blood viscosity in vivo could be ated. The apparent viscosity in vivo was to be considerably lower than that measured o which is in line with several other studies sugito et al 1970. Levy & Share 1953. Whit- & Winton 1933). This is probably due to the nance of the *Fåhraeus-Lindqvist effect* in vessels with consequent reduction of appar scosity. Calculation of apparent blood viscosi- vivo from the very lowest part of the pressure- curves was not reliable with the blood flow ad used. It was therefore not possible to con- a reduction of viscosity in vivo at very low described by others (Djojosugito *et al.* 1970).

en comparing flow rates at different perfusion ures in working and non-working muscle ythemia caused a marked impairment of flow non-working muscle but not in the working e. The flow decrease obtained during non- ng conditions is of the same magnitude that e evaluated by extrapolation from data by n & Richardson (1961) on venous return and vy & Share (1953) on flow in the hind limb ugh rhythmic muscle contractions per se pro- hindrance to flow the rhythmic muscle con- ons will also counteract the disadvantages of sing hematocrit and thereby increased blood sity. In fact the oxygen availability increased an increased hematocrit in the working muscle it decreased in the non-working muscle. The ve effect of rhythmic muscle contractions on ocal blood flow may be due to an increased on gradient. Muscular contractions might ow the radius of small veins and might intermit y empty them of their content of blood. The tying of the veins will decrease the mean post- capillary pressure with a corresponding gain in ef fective perfusion pressure (Folkow et al. 1970 1971). If so rhythmic muscle contractions might increase flow velocity in the capacitance wide-bore vessels where flow and shear rate are proportional ly low and viscosity is proportionally high especial- ly at increased hematocrit.

Beside this postcapillary mechanism another precapillary mechanism is worth considering. Öberg et al. (1975) demonstrated that, in the phar macologically dilated vascular bed and with blood as perfusion medium, *precapillary resistance* and subsequently effective precapillary viscosity were unexpectedly higher than postcapillary resistance and effective postcapillary viscosity. One explana- tion might be that the higher precapillary resistance found with a corpuscular perfusion medium is due to mechanical hindrance and friction occurring when the corpuscular elements of blood are squeezed through the metarterioles and capillaries. In line with this, it might be argued here that heavily exercising muscle facilitates the passage of red cells by squeezing them through the precapillary and capillary network thereby preventing an expected increase of effective precapillary viscosity pro- voked by a denser train of red cells *passing at a* higher hematocrit.

These tentative mechanisms of flow facilitation by muscular rhythmic work at high hematocrits— one precapillary and one postcapillary—suggest dif ferent ways for a small part of the muscular me chanic work to be utilized for blood flow improve ment. Even if the mechanisms of the flow improving effect of rhythmic muscle contractions at high he matocrits are not known and merit further investi- gation the present study clearly shows an increased oxygen availability to the muscular tissue in the polycythemic state. This is in line with earlier find- ings of benefit of a small increase of hematocrit for the aerobic capacity of the organism during physical performance (Ekblom et al. 1972 1976).

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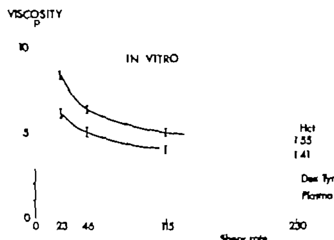


Fig 5 In vitro viscosity data for whole blood Dextran-Tyrode solution and plasma at normal hematocrit and after induced polycythemia (mean ± S E)

after induced polycythemia ($P<0\ 05$) compared to 70±4 and 66±4 ml min⁻¹ 100 g in the working group (n s) At a perfusion pressure of 25 mmHg the flow in the non working group was 35±3 7 ml min 100 g at normal hematocrit and 24±3 0 ml min ' 100 g after polycythemia In the working group the corresponding values were 12±2 and 11±2 ml min 100 g respectively (Fig 2)

In the non-working group blood flow decreased about 35% after red cell transfusion at corresponding perfusion pressures ($P<0\ 05$) with a slightly more pronounced decrease at low perfusion pres sures During exercise there was only a small non significant decrease in flow (5–10%) over the whole pressure range after acutely induced polycythemia

The pressure flow curves for the Dextran Tyrode solution in the working and vasodilated non work ing muscles are shown in Fig 3 The pressure flow curve for the reference solution in the working group is based on values from only 3 dogs since in the additional 3 there were signs of impaired rhythmic contractions and thereby submaximal flow during extracorporeal perfusion As indicated the pressure-flow curve for the Dextran-Tyrode solution is almost identical with that for blood of normal hematocrit in both working and non working muscle

Calculated apparent viscosities in vivo at normal hematocrit and after polycythemia in the working and vasodilated non working muscles are shown in Fig 4 Apparent viscosity in vivo after poly cythemia was 35% higher compared to normal blood with a tendency to an increasing difference at lower perfusion pressures in the non-working muscles ($P<0\ 05$) In the working muscle the r of apparent viscosity in vivo was less th (n s)

The in vitro whole blood viscosity measur revealed highly significant differences b normal and polycythemic conditions at al rates (Fig 5) At shear rates of 23 s⁻¹ the r of whole blood viscosity was about 35 % No change was seen in plasma viscosi polycythemia or after the perfusion procedur

Minute flow of red cells decreased by polycythemia in the non working muscle creased about 25% ($P<0\ 05$) in the working (Fig 6)

DISCUSSION

The experiments were performed in a well vascular bed where vessel geometry alter because of smooth muscle activity were m inducing a pronounced vasodilation of the bed Vasodilation was induced either metab by heavy rhythmical exercise or by i.a. inf papaverine At normal hematocrits blood the papaverine-dilated non-working mu ceeded flow in the exercise-dilated musc about 100% indicating that the twitching ments of the muscles provide a mechanical rance to flow The flow difference is proba to some degree due to an additional vasodil

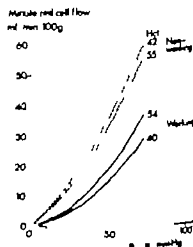


Fig 6 Effect of polycythemia on minute volume cell flow into working (—) and vasodilated non ( ) skeletal muscle

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...ts of physical training on skeletal muscle ...abolism and ultrastructure ...) to 75-year-old men

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ÖRLANDER, J & ANIANSSON A. Effects of physical training on skeletal muscle metabolism and ultrastructure in 70 to 75-year-old men. Acta Physiol Scand 1980 109 149–154 Received 5 Oct. 1979 ISSN 0001-6772. Department of Animal Nutrition, Swedish University of Agricultural Sciences Uppsala, and Department of Rehabilitation Medicine I, Sahlgren s Hospital, Göteborg, Sweden.

The effects of 12-week program of physical training on skeletal muscle (astus lateralis) characteristics in 5 old men were investigated. Heart rates during submaximal bicycle exercise were decreased after training, indicating an improved cardiovascular function. As judged from enzyme activity measurements, the anaerobic capacity as well as the mitochondrial xidative capacity were increased by the training. Fatty acid oxidation capacity remained unchanged while the glycolytic potential tended to be increased, suggesting somewhat different pattern of adaptation as compared to that seen in young subjects. Volume fractions of mitochondria and lipid droplets were unchanged ith training, supporting the view that in old men, increases in oxidative capacity take place within the existing mitochondrial volume. It was concluded, that the aging human skeletal muscle remains trainable and that the training response is similar but possibly not identical to that seen in younger age groups.

Key ords Aging, muscle metabolism, muscle ultrastructure training

well-established regular decline in man s al performance capacity and maximal oxygen with aging (e.g. Åstrand 1960 Åstrand et al Bottiger 1973 Asmussen et al. 1975 Kanstrup blom 1978) was previously thought of as an table process (for ref see Stamford 1972) then, however numerous studies have shown egular physical training produces significant vascular adaptations in middle-aged and persons (Benestad 1965 Saltin *et al.* 1969 ...es 1970 Tzankoff et al 1972 Adams & de 1973 Kiessling et al 1974 Liesen et al 1975 ...ck et al 1976, Suominen et al 1977a 1977b ...y & Shephard 1978 for other ref see Bassey In general, relative improvements similar to in young subjects have been observed In ...ute terms, however the improvement is smal... old age which means that the age decline can... e abolished by training (.g. Saltin t al. 1969)

Considerably less is known about the effects of training on skeletal muscle in old age. Liesen et al (1975) and Suominen et al. *(1977a 1977b) observed* increases in enzyme activities of *energy metabolism* with training in 55 to 70-year-old subjects In a recent cross-sectional study (Orlander *et al. 1978)* we observed no age-related decrease in enzyme activities in sedentary men (age 22–65) and found elevated activities in an older relatively active group This seemed to indicate a persisting trainability in old men, but with a possibly different pattern of adaptation when compared with younger subjects (Örlander et al. 1977 1978).

The present study was designed to further examine the response of the metabolism of the aging human skeletal muscle to physical training. It is part *of* a larger study where effects on strength and muscle morphology are analyzed (Aniansson et al. 1980)

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Enzyme activities in vastus lateralis before and after training

e means ± S.E. for 5 subjects. For full names of enzymes, see Material and Methods

	Enzyme				
	PFK	LDH	HAD	CS	Cyto
	μmol min^{-1}×(g wet wt)$^{-1}$				μmol O_2×min^{-1}×(g wet wt)$^{-1}$
	16.2±3.0	38.8±9.3	8.00±1.74	8.43±1.33	4.91±0.52
	20.4±4.9	57.8±11.9	6.88±0.82	8.87±0.25	6.43±0.53
	>0.05	<0.01	>0.30	>0.70	<0.01

to 46% after training. These values seem hat higher than the 32% observed in the of more active old men, but no definitive sions can be drawn from these limited obser

...SSION

sults of the present investigation indicate that al training produces similar effects in old as ng men, including improved cardiovascular mance as well as metabolic adaptations in al muscle

greement with other studies (for ref. see in-tion), the cardiovascular function was im-d by the present training regimen, as evi-d by the reduced heart rates during submaxi-work. Application of the Åstrand–Åstrand nomogram (Åstrand 1960) suggests a substantial increase in predicted maximal oxygen uptake. The extent of this increase is somewhat uncertain due to low post-training heart rates, and the high age of the subjects.

In addition the strength of the right knee-extensor muscles tended to be increased after training (data not shown). In a larger group of subjects this increase was significant (Aniansson et al. 1980)

Before training, LDH activities were lower and those of HAD higher while PFK, CS and cytox were in the range previously observed in younger age groups (Örlander et al. 1978) (compensation for the differing assay temperatures was made assuming a Q_{10} of 2). The very low LDH activities are, however, in agreement with the findings of Åström et al. (1977) in 65 to 82-year-old men who both during and 4–6 months after acute bacterial infec

Ultrastructural parameters in vastus lateralis muscle fibres before and after training

mparison, data from group of active men, aged 66–76 are included (from Örlander et al. 1978). Values are ± S.E.

		Mitochondria in fibrillar space				Mitochondria in subsarcolemmal space			Lipid droplets
		Volume fraction (%)	Number μm^{-3}	Number Sarcomere	100×Mean volume (μm^3)	Volume fraction (%)	Number (μm^{-3})	100 Mean volume (μm^3)	Volume fraction (%)
(B)	4	2.98 ±0.29	1.81 ±0.48	0.88 ±0.13	2.08 ±0.34	24.1 ±1.7	9.79 ±2.65	3.22 ±0.95	0.43 ±0.12
(A)	4	2.70 ±0.31	2.07 ±0.64	0.95 ±0.18	1.69 ±0.27	20.6 ±2.2	7.92 ±0.94	2.54 ±0.26	0.43 ±0.17
M)	7	2.42 ±0.35	1.30 ±0.19	0.83 ±0.14	2.63 ±0.60	13.1* ±4.4	7.78* ±2.65	2.15* ±0.69	0.46* ±0.16
A)		>0.40	>0.20	>0.50	>0.20	>0.10	>0.60	>0.60	>0.90
M)		>0.20	>0.30	>0.60	>0.60	>0.10	>0.70	>0.70	>0.50
M)		>0.30	>0.10	>0.60	>0.10	>0.20	>0.90	>0.80	>0.90

MATERIAL AND METHODS

Subjects 5 men volunteered to participate in the study None showed any symptoms of cardiovascular or locomotor disease on medical examination Each subject was fully informed of the procedure and possible risks of the experiments before giving his consent The men were all pensioners with various previous occupations and had never participated in any systematic physical training *They currently engaged only in light physical activities* such as going for a walk and were therefore considered sedentary Their age height and weight at the start of the study were 71±1 (70–75) years 173±3 (166–185) cm and 68±4 (62–85) kg respectively (means ± S E ranges within brackets) All of them but one were non-smokers

Training regimen The training was performed under *supervision during 45 min 3 times a week for 12 weeks* Each training workout started with general warm-up exercises such as walking or jogging Then various static and dynamic exercises using body weight and not special equipment as resistance were performed with the main aim to increase muscular strength but also the capacity for prolonged work. The work load during training sessions was estimated to correspond to no more than 70% of the predicted maximal oxygen uptake and was unchanged during the training period The training was considered to be moderately intense for this age group None *of the 5 subjects experienced any medical complications related to* the training Before and after the training period the heart rate response to two submaximal loads (50 and 100 W) on the bicycle ergometer was studied One subject was however unable to complete the *100 W* test before training

Muscle biopsies A needle technique (Bergström 1962) was used to obtain samples from the right vastus lateralis muscle One part of the sample was trimmed oriented mounted in an embedding medium, frozen in isopentane cooled by liquid nitrogen and stored at −80°C for subsequent histochemistry Another part was immediately frozen in liquid nitrogen and stored at −80°C pending enzyme activity determinations A third part was prepared for electron microscopy

Histochemistry Transverse sections (10 μm) were cut with a cryotome and stained for myofibrillar ATPase after alkaline (pH 10.3) or acid (pH 4 4 and 4 6) preincubation (Padykula & Herman 1955 Guth & Samaha 1969 Brooke & Kaiser 1970). F bres were classified as type I (slow twitch) or types II A–C (fast twitch) (Engel 1962 Brooke & Kaiser 1970 Dubowitz & Brooke 1973) The mean number of counted fibres per biopsy was 319 (before) and 324 (after)

Enzyme assays Homogenates were prepared and assays performed as described previously (Örlander et al 1977) except that assays were run at 30°C The 5 studied enzymes chosen to represent the major pathways in energy metabolism were phosphofructokinase (PFK E.C.2.7 1 11) lactate dehydrogenase (LDH E.C.1 1 1.27) *3-hydroxyacyl-CoA dehydrogenase* (HAD E C.1 1 1.35) citrate synthase (CS E.C 4 1.3 7) and cytochrome oxidase (cytox E.C.1 9 3 1) The assay methods used were those of *Shonk & Boxer* (1964) for PFK Bass et al (1969) for *LDH and HAD Srere* (1969) for CS and Whereat et al (1969) for cytox [illegible] estimated according to Lowry et al (1951)

Electron microscopy Small pieces of musc[illegible] were fixed for several hours at 0–4°C in 4% [illegible] buffered glutaraldehyde pH 7 4 washed and [illegible] fixed in 1% veronal-buffered osmium tetroxide for one hour Subsequent treatment and [illegible] lowed the procedure previously described (Örl[illegible] 1977) The calculation of mitochondrial [illegible] volume involves assumptions regarding [illegible] shape and size distribution (Weibel 1969) Th[illegible] tained values are only rough estimates For [illegible] reasons no post training analysis was possib[illegible] subject Therefore only 4 subjects are includ[illegible] ultrastructural results The pre-training values [illegible] subject were similar to those of the others

Statistics Intraindividual and inter-group [illegible] were evaluated by means of the paired and [illegible] *t*-tests respectively A *P* value of less than [illegible] cepted as significant

RESULTS

The training program resulted in significant [illegible] heart rates at both studied submaximal lo[illegible] *50 W a decrease from* 110±5 (mean ±S[illegible] 90±3 beats ×min⁻¹ (*n*=5 *P*<0.01) was [illegible] similar decrease occurred at 100 W from [illegible] *125±9 beats×min* (*n*=4 *P*<0 01).

The percentage type I fibres in the vastu[illegible] lis was 51±4% (mean ± S E.) before and [illegible] *after training* (*P*>0.20) A slight tendency [illegible] an increase in type II A fibres (*P*>0.10) [illegible] from 38±5 to 48±3% (*n*=4) whereas the [illegible] *tions of types* II B and II C fibres were [illegible] (12±6 to 11±4% and 0.5±0.5 to 0.8±[illegible] pectively)

The activities of HAD and CS were [illegible] after training while LDH (49%) and cytox [illegible] increased significantly (both *P*<0 01 Table [illegible] showed a next-to-significant increase [illegible] *P*<0 10) with training The muscle protein [illegible] tration did not change

As shown in Table 2 the volume fraction [illegible] ber and mean volume of skeletal muscle [illegible] chondria were unaffected by the training [illegible] in the fibrillar and the subsarcolemma region [illegible] same was true for the volume fraction of lipid [illegible] lets Furthermore these ultrastructural para[illegible] showed no significant differences when com[illegible] with the corresponding data of a group of [illegible] active 66 to 76-year-old men (Örlander et al [illegible] as shown in Table 2 Lipofuscin occurred in [illegible] the *studied subsarcol* [illegible] *nes before* [illegible]

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tion had only half the LDH activities of young men in the same situations Low LDH activity may be the consequence of a sedentary way of life as suggested earlier in younger men (Bass et al 1976 Órlander et al 1977) but aging processes may also be involved As to the high HAD activities no obvious explanation can be given The plasma level of free fatty acids tends to increase with age (O Sullivan et al 1971) and it is conceivable that substrate induction phenomena might play a role

LDH was seen to increase 49% with training in the present subjects suggesting an elevated anaerobic capacity This observation is in accordance with previous findings in old (Liesen et al 1975 Suominen et al 1977*a*) and young (Örlander et al 1977) sedentary men The 31% increase in cytox activities indicates that the mitochondrial oxidative capacity was augmented after training This agrees with other studies in old subjects (Liesen et al 1975 Suominen et al 1977*a* 1977*b*) as well as middle aged and younger groups (Varnauskas et al 1970 Morgan et al 1971 Gollnick et al 1973 Bergman et al 1973 Kiessling et al 1974 Henriksson 1976 Saltin et al 1976 Bylund et al 1977 Örlander et al 1977) CS and HAD however remained unchanged with training suggesting that the fatty acid oxidation capacity was not markedly influenced On the other hand PFK tended to be increased after training. This agrees with the high PFK activities found in a group of active old men (Örlander et al 1978) and these findings collectively suggest an increased glycolytic capacity with training It thus seems possible that the training response in old men differs somewhat from that in young men with regard to substrate preference The reason for such a difference would not be very clear but one contributing factor might be the elevated blood glucose in the aged (O Sullivan et al 1971) which might be utilized as a primary substrate during exercise The unchanged activity of CS indicates that the citric acid cycle has adequate capacity for supporting an increased aerobic glycolysis In fact the citric acid cycle capacity is known to be closely geared to fatty acid oxidation capacity (Hochachka et al 1977) A consequence of increased carbohydrate utilization during exercise is a more rapid depletion of muscle and liver glycogen which reduces the endurance (cf Fitts et al 1975)

The increased oxidative capacity was not accompanied by a change in the volume fraction of mitochondria Furthermore no significant differences were seen when compared with a[illegible] active old men (Table 2) This supports th[illegible] originally put forward by Kiessling et al (19[illegible] in middle-aged and older persons, an[illegible] oxidative capacity take place within the [illegible] mitochondrial volume in contrast to the[illegible] young men where the mitochondrial vol[illegible] tion increases with training (Kiessling et [illegible] Örlander et al 1977 Bylund et al 1977)

In conclusion the results of the presen[illegible] tion indicate that the aging human skele[illegible] remains trainable at least till the age of[illegible] training response is of a similar magnitude [illegible] possibly of a slightly different nature [illegible] pared with that seen in young age. Furth[illegible] adaptation takes place also in the capil[illegible] system Thus the aged benefit from tra[illegible] similar way as the young

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...cts of metabolic inhibitors on the efflux ...hydroxytryptamine from pancreatic β-cells

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GYLFE, ERIK. Effects of metabolic inhibitors on the efflux of 5-hydroxytryptamine from pancreatic β-cells. Acta Physiol Scand 1980 109: 155–161. Received 11 Oct. 1979. ISSN 0001-6772. Department of Histology Biomedicum, University of Uppsala, Sweden.

The influence of glucose and metabolic inhibitors on the efflux of tritiated 5-hydroxytryptamine (5-HT) was studied in perifused β-cell-rich *ob/ob* mouse islet loaded with trace amounts of ³H-5-hydroxytryptophan. Glucose stimulated exocytotic release of 5-HT but the effect was rapidly inhibited by 2,4-dinitrophenol antimycin A or N-ethylmaleimide. This inhibition was followed by marked stimulation of 5-HT efflux. The later phenomenon was reversible when 2,4-dinitrophenol was used but appeared irreversible with antimycin A or N-ethylmaleimide. The results show that the maintenance of 5-HT within the β-cell depends on energy. It is suggested that both inhibitory and stimulatory effects of 5-HT on insulin secretion depend on release of calcium from the secretory granules after short-circuiting a proton pump across the granule membrane.

Key words: Metabolic inhibitors, serotonin, amine storage, insulin granules

...sulin-releasing β-cells of mice take up both ...oxytryptamine (5-HT Hellman et al. 1972*b* ...ey & Feldman 1977 Gylfe 1978 Lindström ...and its precursor 5-hydroxytryptophan (5-... Gylfe et al. 1973 Gylfe 1978), the latter being converted to 5-HT (Cegrell et al. 1964 ...ark 1971 Tjälve 1971 Gylfe et al. 1973). ... with different techniques have provided evidence for a granular location of 5-HT in ... cells (Jaim-Etcheverry & Zieher 1968 Ek... et al. 1971 Hellman et al 1972*b* Gylfe 1978). ...and other biogenic amines have been reported ...e different effects on insulin secretion some... ...ich may be related to an interaction with cal... ...within the β-granule (Ericson et al. 1977). The ...standing of how 5-HT can affect insulin ...ion would require knowledge about the ...nisms involved in amine accumulation in the ...ules. Studies of 5-HT uptake have shown this ...s to be energy-dependent in the β-cells ...ström 1978). It is evident from the present ...ments with metabolic inhibitors that also the ...enance of 5-HT already present within the ...les depends on energy. It is suggested that ...c amines can both inhibit and stimulate insulin secretion by releasing calcium from the secretory granules.

MATERIALS AND METHODS

Chemicals. DL-5-Hydroxy(G-³H) tryptophan ([³H]5-HTP) was obtained from the Radiochemical Centre Amersham, England. Sigma Chemical Company St. Louis, Mo USA supplied N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes), bovine serum albumin (fraction V), antimycin A, 2,4-dinitrophenol and N-ethylmaleimide. The monoamine oxidase inhibitor N-methyl-N-2-propynyl-benzylamine (Pargyline was a gift from Abbott Laboratories, Chicago USA, whereas dimethylsulfoxide was supplied by Mallinckrodt Chemical Works, St. Louis, Mo., USA. Other reagents were of analytical grade; destilled and deionized water was used.

Medium. Unless otherwise indicated a Krebs-Ringer medium containing 20 mM bicarbonate 20 mM Hepes and supplemented with 1 mM Pargyline and 1 mg/ml albumin was used (Gylfe 1978).

Animals and isolation of islets. Adult *ob/ob* mice were taken from non-inbred colony and used as source of pancreatic islets containing more than 90% β-cells (Hellman 1965). The animals were starved overnight before they were killed by decapitation under ether anesthesia. The pancreas was rapidly excised and immersed in medium lacking albumin and Pargyline but supplemented

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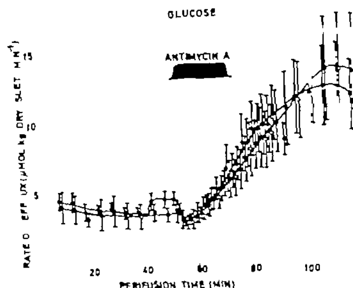


Fig. 2. Effect of antimycin A on 5-HT efflux from pancreatic islets. After loading for 120 min with 16 μM (³H)-HTP in the presence of 20 mM glucose and 1 mM Pargyline the islets were rinsed and perifused with medium containing 1 mM Pargyline 0.1% dimethylsulfoxide and either 3 mM glucose throughout (O) or 3 or 20 mM glucose (●) as indicated by the height of the upper solid bar. Antimycin A (10 μM) was added to the perfusion medium as shown by the lower solid bar. Each point represent the mean of 4 observations ± S E.

occurred in the presence of both 3 and 20 mM ...e about 6 min after addition of 2,4-di...henol or antimycin A and about 10 min after ...on of N-ethylmaleimide. Whereas the late ...latory effect of 2,4-dinitrophenol on 5-HT ...as reversed approximately 6 min after ...ion of the inhibitor stimulation evoked by ...ycin A or N-ethylmaleimide appeared irrever... Although the onset of the late N-ethyl...mide effect was comparatively slow it was ...ved by rapid increase in the efflux rate which ...ed a maximal level twice as high as that ob...d with antimycin A.

...CUSSION

...ll-rich pancreatic islets from ob/ob mice ac...late both 5-HT (Hellman et al. 1972b ...oney & Feldman 1977 Gylfe 1978) and its pre...or 5-HTP (Gylfe et al. 1973 Gylf 1978). A ...titative microchromatographic procedure re...ed that more than 90% of the radioactivity was ...vered as 5-HT after loading of islets in the ...ence of radioactive 5-HTP (Gylf et al 1973) ...es in different species with ultrastructural histochemistry (Jaim-Etcheverry & Zieher 1968) autoradiography (Ekholm et al. 1971) and subcellular fractionation of islet homogenates (Hellman et al. 1972b) have suggested that 5-HT is located to the secretory granules of the pancreatic β-cell. In accordance with such a location a close relationship was observed between 5-HT efflux and insulin secretion (Gylfe 1978). Genuinely amine-secreting cells like chromaffin cells and neurons are thus not the only cells able to concentrate amines. Indeed this *phenomenon seems to be general in cells producing* polypeptide hormones. Three different mechanisms have been suggested to explain the amine-accumulating ability of granules. The amine could be taken up and concentrated by a carrier-mediated process coupled to ATP hydrolysis (Kirshner 1962, Carlsson et al. 1962). Accumulation could also result from the formation of a strong intragranular storage complex between the amine and ATP resulting in amine entry along its concentration gradient (Berneis et al. 1969a–c). The third and most recent proposal involves ATP-dependent generation of an acidic intragranular space and amine entry along the electrochemical gradient (Johnson et al. 1978 Phillips 1978 Phillips & Allison 1978

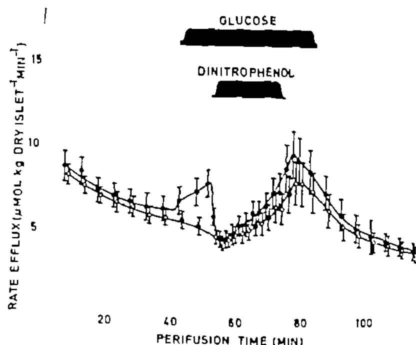


Fig. 1. Effect of 2,4-dinitrophenol on 5-HT efflux from pancreatic islets. After loading for 120 min with 16 μM (³H)5-HTP in the presence of 20 mM glucose and 1 mM Pargyline the islets were rinsed and perifused with medium containing 1 mM Pargyline and either 3 mM glucose throughout (O) or 3 or 20 mM glucose (●) as indicated by the height of the upper solid bar. 2,4-Dinitrophenol (0.3 mM) was added to the perifusion media as shown by the lower solid bar. Each point represents the mean of 4 observations ± S.E.

with 3 mM glucose. In each experiment 20 islets were microdissected from a single pancreas (Hellerström 1964).

Loading and rinsing. The islets were divided into two groups which were incubated for 120 min at 37°C in 100 μl medium containing 16 μM (³H)5-HTP (3 700 Ci/mole) and 20 mM glucose. After loading with radioactivity the islets were rinsed twice for 5 min at 37°C in 5 ml non-radioactive medium containing 3 mM glucose.

Perifusion. The two groups of rinsed islets were transferred to 10 μl perifusion chambers and perifused for 120 min at a flow rate of about 40 μl/min. A fraction collector was used to obtain or 5 min fractions. In one of the perifusion chambers the medium glucose concentration was maintained at 3 mM and in the other it was raised from 3 to 20 mM between 40 and 80 min. The test substances 4-dinitrophenol (0.3 mM), N-ethylmaleimide (0.1 mM) and antimycin A (10 μM) were added to both chambers between 50 and 70 min. Antimycin A was added from a stock solution in dimethylsulfoxide maintaining the final concentration of dimethylsulfoxide at 0.1% throughout the experiment.

Weighing of islets and measurement of radioactivity. After perifusion the islets were freeze-dried (−40°C, 0.1 Pa) overnight and weighed on a quartz fibre balance (Lowry 1953). Samples of each fraction (15 μl) and 100-fold dilutions (15 μl) of the loading media were dissolved in 2 ml toluene Aquasol (1:4 v/v; NEN Chemical GmbH Dreieich, F.R.G.). Radioactivity was measured in a Packard 3375 liquid scintillation spectrometer.

Presentation of results. The efflux data (means ± S.E.) were expressed as mmol 5-hydroxytryptamine equivalents per kg dry islet assuming that the radioactivity of 5-HT was the same as that of 5-HTP in the loading medium.

RESULTS

The effects of glucose and the metabolic inhibitors 2,4-dinitrophenol, antimycin A and N-ethylmaleimide on 5-HT efflux are shown in Figs. When the glucose concentration was raised to 20 mM at 40 min of perifusion there was an enhanced efflux of 5-HT. Statistical analysis of the experiments shown in Figs. 1– revealed the effect was significant at the $P<0.05$ and levels respectively, whereas statistical significance was not reached in the experiments shown in Fig. Exposure of the islets to 0.3 mM 2,4-dinitrophenol (Fig. 1) or 10 μM antimycin A (Fig. 2) almost immediately inhibited glucose stimulated 5-HT efflux whereas inhibition was delayed approximately min when 0.1 mM N-ethylmaleimide (Fig. was added to the perifusion medium. The inhibition of glucose stimulated efflux was followed by a pronounced stimulation of 5-HT washout. The

esent loading conditions (Gylfe 1978). When into account that more than 90% of the ctivity in the efflux medium represents 5-HT 1978) it seems justified to express the re ve efflux in terms of μmoles 5-HT assuming ne specific activity as in the loading medium ne was used throughout the experiments this monoamine oxidase inhibitor enhances cumulation of 5-HT in the islets and con- ily facilitates the subsequent efflux studies 1978).

metabolic inhibitors tested accelerated 5-HT after first having reversed glucose-stimulated efflux. The early effect simply follows from nergy-dependence of exocytosis since glu- timulated 5-HT efflux can be attributed to tosis of β-granules (Gylfe 1978) The late ef- ggests that the retention of 5-HT taken up by eatic β-cells depends on a sustained energy ction though the time-sequence indicates that rocess is less sensitive than the exocytosis ism Thus if there is a mechanism for ic 5-HT binding within the β-granules, which btful it has very low capacity The lack of permanent binding of the amine is also indi- by the gradual loss of 5-HT from preloaded in the absence of metabolic inhibitors (Gylfe

The present data do not allow definite sions about which of the above-mentioned hetical mechanisms accounts for amine ac lation in the β-granules. If a carrier is involved me transport in the β-cells it probably does not the results obtained. Available data on the es of 5-HT uptake (Lindström 1978) and efflux unpublished data) indicate that at the high of 5-HT present in the β-cells after loading, fluxes can be expected to largely represent sion

e relatively rapid effect of the mitochondrial itor antimycin A on 5-HT efflux suggests that ytoplasmic level of ATP is of significance for taining the intragranular 5-HT concentration. most attractive alternative therefore seems to amine accumulation depending on an acidic granular space The presence of a low pH e interior of the β-granule sac might actual- fundamental for maintaining the crystalline ture of the stored insulin. Previous studies indicated that the stored insulin complex is sensitive to pH exhibiting its maximal stabil- pH 6 0 (Coore et al 1969 Howell et al 1969)

However the intracellular pH was estimated to be no less than 7 05 when measuring the uptake of C-labelled 5,5-dimethyloxazolidine-2,4-dione into microdissected islets of *ob/ob* mice (Hellman et al 1972*a*) 2,4-Dinitrophenol can consequently be expected to stimulate 5-HT efflux not only by uncoupling of the oxidative phosphorylation but also by short-circuiting the proton pump in the β-granule membrane Also N-ethylmaleimide could preferentially affect the secretory granules by in- hibiting a proton-translocating ATPase It has been shown in chromaffin cells that this sulphydryl rea- gent inhibits granule ATPase at concentrations lower than those required to inhibit ATPases from other subcellular fractions (Kirshner et al. 1966)

Depending on species concentration of glucose and phase of secretion, 5-HT has been reported to inhibit (Lernmark 1971 Feldman & Lebowitz 1972, Gagliardino et al. 1974) stimulate (Gagliardino et al. 1974 Federspiel et al 1974) or lack effect on the release of insulin (Malaisse 1972). Biogenic amines may affect insulin secretion by acting both at intra- and extracellular sites (Woods & Porte 1976). The effect of intracellular amines may be due to an in- teraction with calcium in the β-granules (Ericson et al. 1977) an idea compatible with the observation that 5-HTP and 5-HT influence the uptake of in- tracellular ^{45}Ca in the β-cells (Lindström & Sehlin 1979). The β-granules contain an exchangeable pool of calcium subject to glucose regulation (Hellman et al 1979). The present data show that 5-HT exhibits a similar sensitivity to metabolic inhibitors as pre viously reported for glucose-stimulated β-cell cal- cium (Hellman 1979). Indeed even the Ca^{2+} uptake into the granules may occur in exchange for protons (Hellman et al 1979). It was suggested that an enhanced concentration of calcium in the submembrane space stimulated insulin secretion only when a certain amount of calcium is present *within* the granule sac (Hellman et al. 1979) Depending on the calcium content in the granules biogenic amines could thus be expected to exert both inhibitory and stimulatory effects on insulin secretion, the common denominator being release of calcium f om the granules after short-circuiting the proton gradient with the amine

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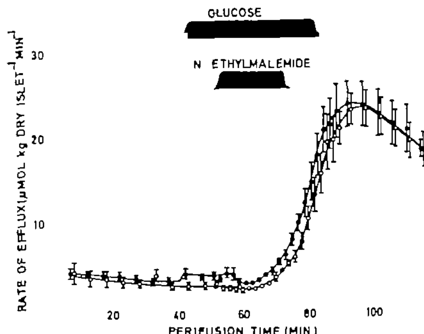


Fig 3 Effect of N-ethylmalemide on 5-HT efflux from pancreatic islets. After loading for 120 min with 16 μM (^{3}H)5-HTP in the presence of 20 mM glucose and 1 mM Pargyline the islets were rinsed and perifused with medium containing 1 mM Pargyline and either 3 mM glucose throughout (O) or 3 or 20 mM glucose (●) as indicated by the height of the upper solid bar N-ethylmalemide (0 1 mM) was added to the perifusion media as shown by the lower solid bar Each point represents the mean of 4 observations ± S E

Aberer et al 1978) None of these alternatives have been ruled out and combinations between them have actually been proposed Johnson & Scarpa (1979) favour a carrier-mediated process energized by the electrochemical gradient

The β-granules differ from chromaffin granules and synaptic vesicles from neurons in not having amines as the major secretory product Exposure of the adrenal medulla to metabolic inhibitors result in an inhibited release of catecholamines (Rubin 1969 1970) indicating that maintenance of the amine within the cell might depend on factors other than the energy requiring uptake alone The presence of more than one process determining the storage of amines in granules is suggested also from the specific storage of one amine in the chromaffin cell or in neurons as opposed to the non-specific accumulation of several amines into the secretory granules of these cells (Shore 1972) In the pancreatic β-cell energy is required for the uptake of 5-HT (Lindström 1978) However nothing is known about the energy requirements for maintaining amines already present in the β-granules

The mechanisms involved in β-granule storage of 5-HT have now been studied by monitoring efflux from preloaded islets during exposure to metabolic inhibitors Inhibitors were selected with different primary actions on metabolism and established effects on the β-cell function at the concentrations employed Both antimycin A blocker of respiration and 2 4-dinitrophenol uncoupler of the oxidative phosphorylation inhibit the glucose stimulated insulin release (Coore & Randle 1964 George et al 1971) A permeability increase of the plasma membrane in terms of sucrose space has been observed after exposing microdissected islets from *ob/ob* mice to 10 μM antimycin A for 60 min (Hellman et al 1973). N-ethylmalemide was taken as representing a penetrating sulphydryl reagent When the same type of islets are exposed to 0 1 mM N-ethylmalemide both the oxidation of glucose and glucose stimulated insulin release are suppressed (Hellman 1979)

The endogenous content of 5-HT in mouse islets has been estimated to approximately 15 μmol wet weight (Hansen & Hedeskov 1977) and only about 5% of the accumulated

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tonin metabolism in neonatal rat brain during yxia and recovery

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HEDNER, T & LUNDBORG P Serotonin metabolism in neonatal rat brain during asphyxia and recovery Acta Physiol Scand 1980 109 163–168, Received 11 October 1978. ISSN 0001-6772. Department of Pharmacology University of Göteborg, Sweden.

Neonatal rats were exposed to 20 or 30 min of total or partial o ygen deprivation. During asphyxia and subsequent recovery the endogenous levels of tryptophan and 5-hydroxytryptamine (5-HT serotonin) were measured. The activity of tryptophan hydroxylase, the first and rate limiting enzyme in the 5-HT synthesis pathw y was studied in vivo by measuring the accumulation of 5-hydroxytryptophan (5-HTP) after inhibition of aromatic L-amino acid decarboxylase with NSD 1015 During asphyxia there was decrease in tryptophan hydroxylase activity in the whole brain and various brain regions studied. The levels of tryptophan, 5-HTP and 5-HT all increased after 30 min of recovery from asphyxia. In the whole brain, 5-HTP and 5-HT levels were normal 2 h after anoxia while tryptophan levels normalized more slowly to reach control values after 6 h. I the regional brain study the 5-HTP levels returned quickly to control levels after asphyxia in the striatum and midbrain but not in the brainstem and hemispheres regions. The whole brain 5-HTP and 5-HT levels did not differ from controls 24 and 48 h after the asphyxia. Although the neonatal nervous system exhibits great resistance to asphyxia, the metabolism of the neurotransmitter 5-HT is affected already during short period of asphyxia and subsequent recovery As 5-HT is ascribed important neurotransmitter functions, this might be relevant to the neurological sequelae of human asphyxia neonatorum.

Key ords Experimental study neonatal asphyxia, 5-HT CNS neurotransmitter

oxytryptamine (5-HT) has been ascribed a r of functions as neurotransmitter in the l nervous system as for example, control of induction repression of sexually active be- central regulation of body temperature and pation in number of psychic functions rson 1964 Reid et al. 1968 Jouvet 1969).

ent studies have demonstrated that 5-HT es are present in the brain relatively early the ontogenetic development. By means of hemical techniques, 5-HT can be visualised in odies in the raphe nuclei as well as in the l processes at the base of the midbrain already the second trimester of the rat (Olson & 1972). The development of the serotoniner stem is from this point accelerating to reach ture state in the 5th or 6th postnatal week in t (Bennet & Giarman 1965 Schmidt & Sanders-Bush 1971 Deguchi & Barchas 1972 Loizou 1972, Tissari 1973)

Previous studies in our laboratories have demonstrated that acute 12% hypoxia in unanes thetized developing rats is accompanied by a decreased in vivo rate of brain tryptophan hydroxylation. This effect appeared to be somewhat less pronounced in the early postnatal period than later in development (Hedner et al. 1977a) A more severe hypoxia did only in a few cases result in a further decrease in enzyme activity (Hedner et al 1978). When, however the period of 12% hypoxia was prolonged to 6 h this resulted in a normalisation of enzyme activity or In the neonatal rats, even an increased activity compared to controls (Hedner et al 1977b).

Since the neonatal rats in this and certain other respects seem to react differently than older ani-

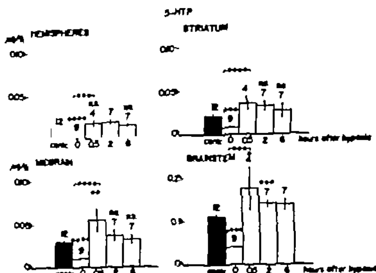


Fig. 3. 5-HTP levels in brain regions of 4-day-old rats during 30 min 6% hypoxia and recovery. Animals were injected subcutaneously with NSD 1015, 100 mg/kg, 30 min before sacrifice. Shown are means ± S.E. Figures indicate the number of observations. $P<0.05$ $^{**}P<0.01$ $^{***}P<0.005$ $P<0.001$. When not specifically shown, significance is indicated versus controls.

...ran was dissected out and immediately frozen on ...). In the regional brain study the brain was re... and a "functional dissection" (Carlsson & ... et 1973) was performed on an ice-cold glass ...o, (1) striatum including corpus striatum and lim... ...brain, (2) hemispheres including hippocampus, ...cephalon ("midbrain") and (4) lower brain stem. ...um ...as removed and rejected. Most of the 5-HT ...ng ser... cell bodies are found in part (4). The ...mples ...are stored at −70°C in ... freezer in no case ...e than 2 months. For the biochemical analyses, 4 ...rains or 10 brain parts were weighed and pooled ...sample. The samples were homogenized in 25 ml ...ubes, containing 10 ml perchloric acid, 0.2 ml 10% ...and 0.1 ml 5% $Na_2S_2O_5$. The extracts were purified ...rongly acidic cation exchange column (Dowex ...and analysed for tryptophan, 5-HTP and 5-HT ...& Magnusson 1970, Bedard et al. 1972, Atack & ...rt 1973).

...tical analyses were performed using Student' ...lues ...ere considered significant when $P<0.05$.

...LTS

...phan levels

...exposure to nitrogen, whole brain tryp... levels did not change in the 1-day-old ani... Fig. 1). In the recovery phase, there was a ...cant increase in tryptophan levels compared ...trol animals after 0.5 and ... h ($P<0.001$ and $P<0.05$ respectively). Control values were reached after 6 h. An increase in tryptophan levels ($P<0.05$) compared to controls was however again observed 48 h after the anoxic period.

In the regional brain study tryptophan levels tended to increase during hypoxia and subsequent recovery, however reaching significance only in the brain stem region 2 h after hypoxia (Table 1).

5-HTP levels

At the end of the 20 min anoxia there was a decrease in the amount of whole brain 5-HTP accumulated after NSD 1015 ($P<0.05$) in the newborn animals (Fig. 2). After 30 min in the recovery phase 5-HTP accumulation increased approximately twofold ($P<0.001$). After this peak value a successive decline in the rate of 5-HTP accumulation was observed, reaching control values after ... h and subnormal values ($P<0.05$) 6 h after the nitrogen exposure. No changes compared to control animal were observed after 24 and 48 h.

In all brain regions, 5-HTP accumulation after NSD 1015 similarly decreased during hypoxia (Fig. 3). An increase in 5-HTP accumulation was observed in all regions either 0.5 or 2 h after the 6% hypoxia. At 6 h after the hypoxia control levels were reached in all regions except for the brain stem

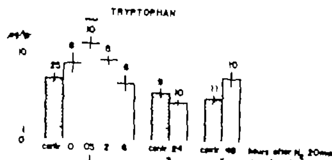


Fig 1 Tryptophan levels in rat brain during nitrogen anoxia and recovery Shown are means ± S E Figures indicate the number of observations $P<0.001$ $P<0.05$ n s = not significant

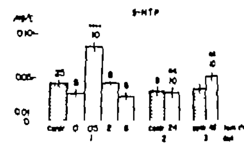


Fig 2 5-HTP levels in rat brain during anoxia and recovery Animals were injected subcutaneously NSD 1015 100 mg/kg 30 min before sacrifice. Means ± S E. Figures indicate the number of observations $P<0.001$ $P<0.05$ n s not significant

mals to oxygen deprivation we have focussed our attention on the early postnatal period and designed this investigation so as to follow the 5-HT metabolism in the neonatal rat brain during asphyxia and the following recovery period

METHODS

Pregnant Sprague-Dawley rats (Anticimex Stockholm) were obtained and housed in the department The animals were kept in separate boxes under regulated dark–light conditions (light period 6 a m –6 p m) The time of delivery was noted within 12 h

The neonatal (24–36 h or 4 days old) rats were kept with their mothers the first day or days of life Thereafter they were transferred to a 24 litre plastic cage Experimental animals were rendered anoxic for 20 min with 100% N_2 or hypoxic for 30 min with 6% O_2–94% N The gas mixture was passed through the plastic cage at a rate of about 4 l/min Control animals were under similar conditions exposed to room air The cages were kept on a preheated operating table (35–36°C) Room temperature was 22°C

After anoxia or hypoxia (or corresponding period for the control animals) some animals were killed immediately whereas the others were returned to their mothers and allowed to breathe room air (see below) All experiments started around 4 h after the onset of light.

Some of the neonatal rats (experimental and controls) were intended for brain analysis of tryptophan and 5-hydroxytryptophan (5-HTP) after inhibition of aromatic L-amino acid decarboxylase These animals received a subcutaneous injection of NSD 1015 (3-hydroxybenzylhydrazine HCl synthetized in this laboratory by Lindberg) 100 mg/kg, dissolved in saline 30 min before they were killed These animals as well as the animals analysed for brain 5-HT were killed immediately after oxygen deprivation (or corresponding period in air) or 0 5 6 24 or 48 h after exposure

In a separate experiment aiming to study the impulse induced release of 5-HT during hypoxia 4 day old rats were given a s c injection of H22/54 (α-propyldopacetamide AB Hässle Mölndal) 500 mg/kg at the start of the experiment and then rendered hypoxic (6% O_2–94% N_2) After 1 or h in the hypoxic environment the animals were *decapitated* and the whole brain analysed for 5-HT Zero control animals were injected with normal saline

In all experiments the animals were decapitated

Table 1 *Tryptophan levels in brain regions of 4 days old rats during 30 min of 6 % hypoxia and recovery in room air*

Shown are means ± S E Figures within bracket indicate the number of observations Significance is indicated vs control levels $P<0.05$ n s = not significant

	Hemispheres	Striatum	Midbrain	Brain stem
Control	5 7±0 44 (1)	6 5±0 4 (1)	6 5±0.39 (1)	6.2±0.34 (1)
Hypoxia	6 6±0.54* (9)	7 6±0 65* (9)	7 4±0 59* (9)	7 1±0 68* (9)
0.5 h after hypoxia	6 2±0.88* (7)	7.3±0 93* (7)	7 5±0 70* (6)	7 4±0 66* (5)
2 h after hypoxia	7 2±1 1* (7)	8.5±1 30* (7)	7 6±0 91 (7)	7 9±0 89* (7)
6 h after hypoxia	5 9±1 14 (7)	6 9±1 18* (7)	7.5±0 85* (7)	7 0±0.79* (7)

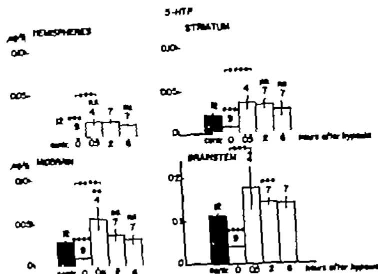


Fig. 3. 5-HTP levels in brain regions of 4-day-old rats during 30 min 6% hypoxia and recovery. Animals were injected subcutaneously with NSD 1015 100 mg/kg, 30 min before sacrifice. Shown are means ± S.E. Figures indicate the number of observations. $P<0.05$ $^{**}P<0.01$ $^{***}P<0.005$ $^{****}P<0.001$. When not specifically shown significance is indicated versus controls.

...rats was dissected out and immediately frozen on ...), In the regional brain study the brain was re... and "functional dissection" (Carlsson & ... et 1973) was performed on an ice-cold glass ... (1) striatum including corpus striatum and lim... ...brain, (2) hemispheres including hippocampus, ...cephalon ("midbrain") and (4) lower brain stem. ... was removed and rejected. Most of the 5-HT ...ing nerve cell bodies are found in part (4). The ...mples were stored at −70°C in freezer in no case ... than 2 months. For the biochemical analyses, 4 ...rams or 10 brain parts were weighed and pooled ... sample. The samples were homogenized in 25 ml ...ubes, containing 10 ml perchloric acid, 0.2 ml 10% and 0.1 ml 5% $Na_2S_2O_5$. The extracts were purified ...rongly acidic cation exchange column (Dowex ... and analysed for tryptophan, 5-HTP and 5-HT ... & Magnusson 1970, Bédard et al. 1972, Atack & ... al 1973).

...tical analyses ... ere performed using Student's *t* ... ...ences were considered significant when $P<0.05$.

...ULTS

...phan levels

...g exposure to nitrogen, whole brain tryp... ...n levels did not change in the 1-day-old ani... (Fig. 1). In the recovery phase there was a ...icant increase in tryptophan levels compared ...ntrol animals after 0.5 and 2 h ($P<0.001$ and $P<0.05$ respectively). Control values were reached after 6 h. An increase in tryptophan levels ($P<0.05$) compared to controls was, however, again observed 48 h after the anoxic period.

In the regional brain study tryptophan levels tended to increase during hypoxia and subsequent recovery, however reaching significance only in the brain stem region 2 h after hypoxia (Table 1).

5-HTP levels

At the end of the 20 min anoxia there was a decrease in the amount of whole brain 5-HTP accumulated after NSD 1015 ($P<0.05$) in the newborn animals (Fig. 2). After 30 min in the recovery phase 5-HTP accumulation increased approximately twofold ($P<0.001$). After this peak value a successive decline in the rate of 5-HTP accumulation was observed reaching control values after 2 h and subnormal values ($P<0.05$) 6 h after the nitrogen exposure. No changes compared to control animals were observed after 4 and 48 h.

In all brain regions 5-HTP accumulation after NSD 1015 similarly decreased during hypoxia (Fig. 3). An increase in 5-HTP accumulation was observed in all regions either 0.5 or 2 h after the 6% hypoxia. At 6 h after the hypoxia control levels were reached in all regions except for the brain stem

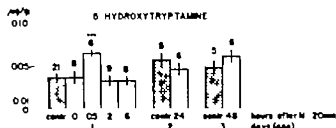


Fig 4 5-hydroxytryptamine levels in rat brain during nit rogen anoxia and recovery Shown are means ± S E. Figures indicate the number of observations $P<0\ 005$ n.s. = not significant.

region where the increased 5 HTP accumulation persisted

5-HT levels

Similarly to tryptophan and 5 HTP 5-HT levels in the 1 day old animals had increased by about 75% 30 min after the nitrogen exposure ($P<0\ 005$) (Fig 4) No other changes in 5-HT levels compared to control animals were noted

After blockade of the tryptophan hydroxylase with H22/54 no difference in the disappearance rate of 5-HT was noted between the hypoxia and control groups during 6% O_2 for 1 and 2 h in the 4 days old animals (Fig 5)

DISCUSSION

Tryptophan hydroxylase is the first and probably rate limiting enzyme in serotonin formation in the brain (Costa & Neff 1970) In the rat brain the regional distribution of tryptophan hydroxylase ac tivity parallels that of 5-HT (Schmidt & Sanders Buch 1971 Deguchi & Barchas 197?) During de- velopment the increase in serotonin levels in the brain seems to parallel the increase in tryptophan hydroxylase activity (Bennet & Giarman 1965 Tis sari 1973)

The second enzyme in the serotonin synthesis pathway aromatic L amino acid decarboxylase can effectively be blocked by NSD 1015 (Carlsson et al 1972) After blockade the intermediate amino acid 5-HTP accumulates in a linear manner during the first 30 min Thus analyzing the amount of 5-HTP accumulated after NSD 1015 can be used as an accurate method ty study serotonin synthesis (Carlsson et al 1972)

Most of the oxygen consumed by the mammal brain is used for energy production in the process of oxidative phosphorylation Apart from the fraction of the total oxygen requirement is hydroxylation reactions such as e g in the thesis of 5-HT and other monoamine mitters

Previous studies from this laboratory have that oxygen deprivation causes a decrease synthesis of the transmitters 5-HT dopamine and noradrenaline (NA) in the neonatal (Hedner et al 1977*a* *b* 1978). In those 6–12% environmental hypoxia for 30 min inhibited 5-HTP accumulation in the brain present study oxygen deprivation re duced tryptophan hydroxylase activity by 30% compared to controls All brain ap peared to be similarly affected No change levels of 5-HT or the precursor amino acid tophan were noted during oxygen depriv

The formation of 5-HTP from tryptophan quires the presence of molecular O_2 in the brain (Carlsson et al 197?) as well as in the rat brain (Hedner et al 1977*a* *b* 197 explains the decrease in 5-HTP accumula ing hypoxia in the present study The O_2 r ment of the major 5-HT degrading e monoamine oxidase may also explain the decrease in 5-HT levels during hypoxia

The decrease in whole brain 5-HTP level anoxia was followed by a sharp rise 6 h anoxia. At this point in the recovery phase was also an increased availability of prec whole brain tryptophan levels were raised troptophan levels declined slowly for 6 h 5 HTP accumulation after NSD 1015 retu

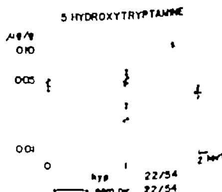


Fig 5 5 Hydroxytryptamine disappearance ... day old rat whole brain during 6% hypo ... hours H 22/54 (α-propyldopacetamide) 500 mg injected 1 and h bef ... respecti

after 2 h and even reached subnormal values hypoxia.

regional brain study for technical reasons ned on 4 days old rats a similar course of for the levels of tryptophan and 5-HTP was s in the whole brain. However in the brain gion where most of the 5-HT nerve cell are located the increased 5-HTP levels per or the whole 6 h observation period after a. The explanation for this discrepancy be different brain regions can only be speculated One possible explanation may be that the nerve cell bodies are more sensitive to a than other parts of the 5-HT neuron.

yptophan hydroxylase is not fully saturated a substrate tryptophan under normal condi- Carlsson & Lindqvist 1978) the increase in levels in the brain may in part reflect the ed availability of tryptophan. On the other earlier studies have shown that tryptophan e relatively ineffective in increasing 5-HT ses in the neonatal rat brain (Bourgoin et al. other causes may also well be responsible for served changes in 5-HTP accumulation after a. Such exogenous factors may for example -changes, changes in enzyme activity s in oxygen availability or alterations in metabolism

a consequence of the increased 5-HTP ac ation, brain 5-HT levels also increased during mediate recovery from anoxia, but were y restored to normal later in the recovery No persistent effects on the brain serotonin olism were observed 48 h after the hypoxia ure.

of the changes caused by hypoxia on energy olism such as ATP and phosphocreatine in ain seem to be readily reversible if the normal nation and arterial circulation is restored ver to understand the mechanisms responsi- the brain pathology produced by asphyxia, lucidation of early metabolic and structural tions occurring in the affected tissue is of con- able importance. As recent results from our atory indicate that permanent alterations in serotonin metabolism can be caused by en depri ation (Hedner et al 1979) this field of rch warrants further investigation.

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...cholamine metabolism in neonatal rat brain ...ng asphyxia and recovery

...AS HEDNER and PER LUNDBORG

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HEDNER, T & LUNDBORG P Catecholamine metabolism in neonatal rat brain during asphyxia and recovery Acta Physiol Scand 1980 109 169–175. Received 11 Oct. 1978. ISSN 0001-6772. Department of Pharmacology University of Göteborg, Sweden.

Infant rats were subjected to various degrees of asphyxia—1-day-old rats were exposed to 100% N_2 for 20 min and 4-day-old rats were exposed to 94% N_2–6% O_2 for 30 min. The activity of tyrosine hydroxylase the first and rate limiting enzyme in the catecholamine (CA) synthesis, was studied in vivo in whole brain and in various brain parts by measuring the accumulation of dihydroxyphenylalanine (DOPA) after inhibition of aromatic L-aminoacid decarboxylase with NSD 1015 Measurements of the endogenous levels of tyrosine, dopamine (DA) and noradrenaline (NA) were also made during asphyxia and recovery During asphyxia there was decrease in DOPA accumulation in the whole brain of the 1-day-old animals and in the striatum, midbrain and brainstem regions of the 4-day-old rats In the recovery phase both tyrosine and DOPA levels in the whole brain of the 1-day-old animals increased approximately 2-fold 30 min after anoxia. In the regional brain study significant increase compared to controls was only noted in the striatum region. After 2 h, tyrosine and DOPA accumulation had reached normal or subnormal levels In the 1-day-old rats there were no changes in whole brain DA or NA levels during anoxia and recovery except for an increase in DA levels noted after 6 h in the recovery phase. The disappearance rate of DA but not NA during hypoxia was retarded in the whole brain of the 4-day-old animals after H 44/68 (α-methyltyrosine), indicating a reduced nerve impulse activity in central dopaminergic but not noradrenergic nerves. The catecholamine neurotransmitters DA and NA are ascribed lot of important functions in the mature brain. As hypoxia or anoxia cause profound changes in the metabolism of these transmitters during the neonatal period, this might be relevant as a factor underlying early childhood behavioural or neurological disorders.

Key words Neonatal brain, asphyxia, dopamine noradrenaline

...mature and developing nervous system of ...l and neonatal animals exhibits a marked toler... to anoxia and hypoxia. This seems to be a ...tion of the low energy requirements of the ...atal brain (Himwich et al 1942, Jilek 1970 ...let 1976) In spite of these protective mech...sms, anoxia is followed by several cerebral ...tabolic responses (Vannucci & Duffy 1976). It ...recently been reported that the catecholamine ...tabolism is affected already during relatively ...derate hypoxia in the adult as well as in the ...born rat brain (Davis & Carlsson 1973 Hedner ...al. 1977*a*). The behavioural effects caused by ...xia, as suppression of locomotor activity and ...n of conditioned avoidance response seem ...lated to the decrease observed in catecholamine metabolism (Brown et al. 1973 Brown & Engel 1973) These findings may explain why brain function during oxygen deprivation fails before there is any significant change in energy metabolism (Duffy et al. 1972) In previous studies from this laboratory we have shown that the activity of tyrosine hydroxylase the first and rate limiting enzyme in the catecholamine synthesis pathway decreases in the rat brain during 30 min of 12% hypoxia (Hedner et al. 1977*a*) The relative decrease appeared to be similar for the various ages studied *Additionally* only a slight further decrease in enzyme activity could be noted after decreasing the O_2 levels below 12% (Hedner et al 1978). However when xtending the hypoxic period, enzyme activity tended to return to normal after 6 h in the

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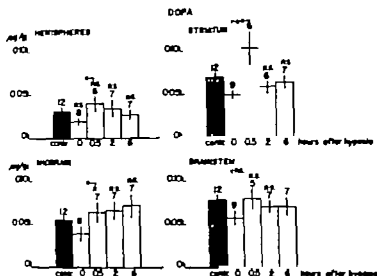


Fig. 3. DOPA levels in brain regions of 4-day-old rats during 30 min 6% hypoxia and recovery. Animals were injected subcutaneously with NSD 1015 100 mg/kg, 30 min before sacrifice. Shown are mean ± S.E. Figures indicate the number of observations. ** $P<0.01$ $P<0.05$ n.s. = not significant. When not specifically shown, significance is indicated versus controls.

...phere) was quickly removed and dissected on an ...l glass plate into the following parts: (1) striatum ...ing corpus striatum and limbic forebrain, (2) hemi... ...s including hippocampus, (3) diencephalon ("mid...") and (4) lower brain stem. Cerebellum was iden... ...and discarded. NA is the predominating ...olamine in parts (2), (3) and (4) and DA in part (1). ...tails of the dissection procedure, see Carlsson & ...vist (1973). The samples were stored in freezer at ...y, in no case for more than 2 months. Four whole ...from the 1- and 4-day-old animals and 10 brain parts ...he 4-day-old animals were pooled for the biochemi... ...alyses. The brain samples were homogenized in 10 ...N perchloric acid containing 5 mg $Na_2S_2O_5$ and 20 ...DTA. The extracts were purified on a strong cation ...nge column (Dowex 50-X-4) (Atack & Magnusson ...Kehr et al. 1972) and analysed spectrofluorimetrical... ...tyrosine and DOPA or DA and NA according to ...iously described techniques (Waalkes & Udenfriend ...Bertler et al. 1958, Atack & Magnusson 1970, Kehr ...1972, Atack 1973).

...tistical analyses were performed using Student's *t*... ...P-values larger than 0.05 were considered not sig... ...ant.

...ULTS

...ry behaviour

...he end of the nitrogen exposure the breathing of ...1-day-old animals was found to be irregular and infrequent. However, the mortality during the 20 min exposure was very low, less than 5%. The 4-day-old animals exposed to 6% hypoxia were less affected and no mortality occurred during or after exposure.

Tyrosine levels

The influence of nitrogen exposure on the brain tyrosine levels in 1-day-old animals is presented in Fig. 1. The tyrosine levels did not change during the nitrogen exposure. A two-fold increase ($P<0.001$) compared to control values was, however, observed 0.5 h after exposure. Thereafter the tyrosine levels declined to normal after 2 h and after a further decline they were found to be below control values ($P<0.005$). No difference was found between the experimental and control groups at 48 h after exposure. In the regional brain study performed in 4-day-old animals (Table 1) a significant decrease in tyrosine levels during hypoxia was only noted in the brain stem region ($P<0.05$). During the recovery period tyrosine levels increased significantly in the hemispheres, striatum and the brain stem regions. The maximal increase was 27% in the hemispheres, 63% in the striatum and 63% in the brain stem region.

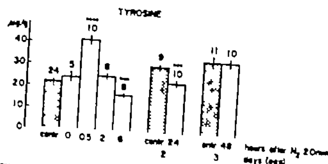


Fig 1 Tyrosine levels in 1-day-old rat brain during 20 min nitrogen anoxia and recovery Shown are mean ± S E Figures indicate the number of observations. $P<0.001$ $P<0.005$ n.s = not significant.

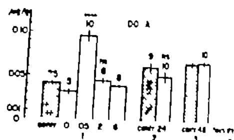


Fig 2 DOPA levels in 1-day-old rat brain during nitrogen anoxia and recovery Animals were [illegible] cutaneously with NSD 1015 100 mg/kg [illegible] sacrifice Shown are mean ± S E. Figures [illegible] number of observations $P<0.001$ [illegible] not significant

neonatal animals but not in the older age group (Hedner et al 1977*b*) These findings have prompted us further to investigate the alterations in catecholamine metabolism in the neonatal rat brain during anoxia or hypoxia and recovery

METHODS

Pregnant Sprague-Dawley rats (Anticimex Stockholm) were obtained at the 16th–18th day of gestation The animals were housed in the department under regulated dark–light conditions (light period 6 a.m.–6 p.m) and the time of birth was noted within 12 h. The neonatal animals were exposed to a hypoxic environment in a sealed 2 litre plastic cage 1-day-old rats—100 %N for 20 min and 4-day-old rats—94 %N_2–6 %O_2 for 30 min 1 or 2 h After this exposure some neonates were returned to their dam allowed to breathe room air All experiments were started around 4 h after onset of light The nitrogen gas was passed through the cage via inlet and outlet holes at a rate of about 4 l/min Control animals were kept in a similar box separated from the mother exposed to room air The cages were kept on a preheated (35-36°C) [illegible] room temperature was 27°C

At various intervals after the hypoxic exposure [illegible] perimental and control animals were analysed [illegible] content of either tyrosine and DOPA or DA and [illegible] min before sacrifice all animals in which the [illegible] or brain parts were analysed for tyrosine and DOPA injected subcutaneously with NSD 1015 100 mg/kg hydroxybensylhydrazine HCl synthetized [illegible] tory by Dr Per Lindberg) The animals analysed [illegible] DA and NA did not receive any injections

In a separate experiment 4-day-old animals [illegible] with H 44/68 (α-methyltyrosine) 250 mg/kg s.c. [illegible] (zero controls) The H 44/68 treated animals [illegible] mediately exposed to 6 %O_2–94 %N or room air [illegible] h After this period the animals were decapitated analysed for whole brain DA and NA

The neonatal animals given NSD 1015 were [illegible] mediately after 0.5 6 4 or 48 h after the [illegible] 100 %N exposure or immediately after 0.5 2 or 6 [illegible] the 94 %O_2–6 %N exposure After the decapitation whole brain of the 1-day-old animals was dissected and immediately frozen on solid CO_2. In the [illegible] study of the 4-day-old rats the whole brain [illegible]

Table 1 *Tyrosine levels in brain regions of 4-day-old rats during 30 min of 6 % hypoxia and recovery room air*

Shown are mean ± S E. Figures within brackets indicate the number of observations Significance is indicated [illegible] control levels. $P<0.001$ $P<0.005$ $P<0.01$ $P<0.05$ n s = not significant

	Hemispheres	Striatum	Midbrain	Brain stem
Control	28 3±1 40 (10)	33 4±1 81 (1)	35 0±4 28 (8)	33 7±1 66 (11)
Hypoxia	26 7±1 9 (7)	29 5±2 76 (9)	31 9±2.33 (7)	28 1± 01 (9)
0.5 h after hypoxia	35 7±2.49* (7)	49 0±5.34 (7)	46 0±3 40* (6)	44 0±3 94 (5)
2 h after hypoxia	35.2±3 71 (7)	54 4±5.22 (6)	49 0±9 33 (7)	55 0±6 84 (7)
6 h after hypoxia	35 9±2 36 (7)	41 3±3 76 (7)	37 7± .58* (7)	46.5±6.58* (7)

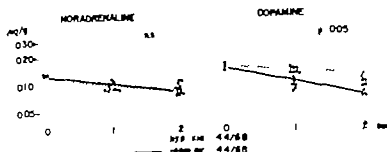


Fig. 6. Dopamine and noradrenaline disappearance curves in 4-day-old rat whole brain during 6% hypoxia for 1 and 2 h. H 44/68 (α-methyltyrosine) was injected 1 and 2 h before sacrifice, respectively.

...A accumulates in a linear manner. Measuring ...A can thus be used to study the in vivo activity ...osine hydroxylase (Carlsson et al. 1972).

...eral cerebral metabolic responses occur during ...nd after acute anoxia in newborn animals. ...rmed and potential energy stores such as ...gen, glucose, phosphocreatine and total adenine ...ucleotides decrease during anoxia, followed by a normalization or even an increase during ...ecovery phase (Berlet 1976). On the other ...lactate increases in the brain during anoxia, ...result of cerebral glycolysis as well as uptake ...extracerebral sources. During recovery a successive decrease in brain lactate concentration ...place (Vannucci & Duffy 1976).

...though the cerebral metabolic response to ...is extensive, brain function fails before there ...any significant changes in ATP or phospho...ine (Duffy et al. 1972). This phenomenon may ...at in part be explained by the decreased syn...s of catecholamines, which has been found to ...already at a moderate hypoxia or short anoxia ...m & Carlsson 1973, Hedner et al. 1977 b...). During 20 min of anoxia, the DOPA accumulation after NSD 1015 treatment was found to ...ecreased to about the same extent as has previ...y been demonstrated after a moderate hypoxia ...2% O_2 during 30 min (Hedner et al. 1977 b...5). In the present study 6% hypoxia significant...reduced brain DOPA accumulation after NSD ...5 in all the brain parts studied except for the ...isphere region. Hence the changes in CA ...abolism caused by short term oxygen depriva...n are evident in the neonatal rat brain at a point ...en other metabolic patterns appear to be undis...rbed.

After the anoxic period there was a marked increase in whole brain DOPA accumulation after NSD 1015, which may be the result of a variety of possible factors, such as increased oxygen tension, pH changes, arteriole shunting, alterations in energy metabolism or changes in neuronal transport or availability of precursors. The events in the various brain regions after hypoxia were similar but not identical. There was a more marked increase in tyrosine hydroxylase activity in the striatum region compared to the other areas 30 min after hypoxia. As for the whole brain the regional increase in tyrosine hydroxylase was short lasting and not detectable during further measurements 2 and 6 h after hypoxia. Hence these results indicate that the DA and NA neurons in the neonatal brain react similarly as the events occurring in a DA predominated area (striatum) almost parallel to those occurring in the NA predominated areas (hemispheres, midbrain).

During the postanoxic phase the tyrosine levels in the whole brain as well as in the various brain regions increased and decreased in a way similar to that of DOPA. Although tyrosine hydroxylase is normally considered to be almost fully saturated with its precursor amino acid (Carlsson & Lindqvist 1978), increased levels of tyrosine might under certain conditions such as asphyxia be at least partly responsible for the increased levels of DOPA observed. As the enzyme tyrosine hydroxylase requires molecular oxygen for the synthesis of the new hydroxyl group of DOPA, the variations in DOPA accumulation observed during and after the anoxic period seem to indicate a decreased rate of catecholamine synthesis during anoxia and an increased rate in the immediate postanoxic phase.

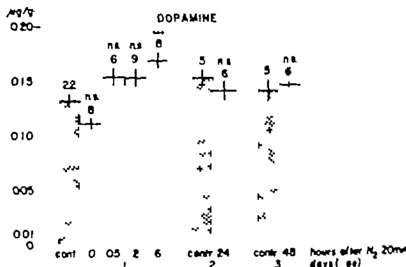


Fig 4 Dopamine levels in 1-day-old rat brain during 20 min nitrogen anoxia and recovery Shown are mean ± S E. Figures indicate the number of observations. ** $P<0.005$ n.s = not significant

DOPA levels

The whole brain DOPA levels observed in the 1 day-old animals after treatment with NSD 1015 were significantly below control values ($P<0.05$) immediately after 20 min in the 100% N_2 environment (Fig 2) 30 min after the N_2 exposure the DOPA levels had increased markedly ($P<0.001$) compared to the control group From 2 h on after the exposure the DOPA levels had returned to normal

In the various brain regions apart from the hemispheres in the 4-day-old rats (Fig 3) 30 min of hypoxia induced a significant decrease in DOPA accumulation after NSD 1015 During the first 30 min of recovery from hypoxia DOPA levels increased significantly compared to control levels only in the striatum region No changes compared to controls were noted 2 and 6 h after the 6% O_2–94% N_2 exposure for 30 min

Dopamine and noradrenaline levels

No change in NA levels was found at any interval studied after N_2 exposure (Fig 5) Neither did the DA levels change significantly during anoxia or recovery except at 6 h after the N_2 exposure where a slight increase compared to the control group was observed (Fig 4)

Administration of H 44/68 caused a decline in DA and NA levels under control conditions During the 2 h exposure to 6% O_2–94% N_2 the disappearance rate for brain DA was reduced ($P<0.05$) (Fig 6) compared to controls NA disappearance r[illegible] brain did not differ between the control [illegible] mental groups (Fig. 6)

DISCUSSION

The hydroxylation of tyrosine to DOPA is the [illegible] and rate limiting step in the catecholamine b[illegible] thesis (Udenfriend 1966) Tyrosine hydroxyl[illegible] tivity has been detected in the rat brain [illegible] gestation and tyrosine hydroxylase is [illegible] first enzymes to appear in the catechol[illegible] biosynthesis pathway (Coyle & Axelrod [illegible] NSD 1015 is a potent inhibitor of the arom[illegible] amino acid decarboxylase in the brain. It [illegible] demonstrated that during the first 30 min aft[illegible] 1015 administration the intermediate [illegible]

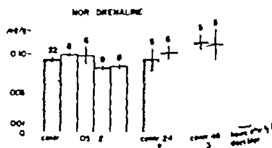


Fig 5 Noradrenaline levels in 1-day-old rat brain [illegible] 20 min nitrogen anoxia and recovery Shown are me[illegible] S E. Figures indicate the number of observations [illegible] not significant

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Contrasting to these findings no major changes were observed in the endogenous whole brain levels of DA and NA After synthesis inhibition the DA levels during hypoxia decreased more slowly than in the control situation Thus the firing rate of DA neurons is inhibited during hypoxia in the neonatal brain This inhibition of firing during hypoxia was not present for the NA neurons

Although the degradation of CA was not investigated in the present study we can predict that during hypoxia there probably is a decrease in the degradation rate compensating for the decrease in synthesis rate This is most certainly true for the NA neurons as neither the endogenous levels of the transmitter nor the firing rate of the neurons were altered during hypoxia In fact studies in adult animals have demonstrated that monoamine oxidase but not catechol O-methyl-transferase is inhibited during oxygen deprivation (Davis & Carlsson 1973)

The changes occurring in the DA and NA neurons during and after a brief period of anoxia or hypoxia appeared to be relatively shortlasting and generally they could not be detected 24 and 48 h after the anoxic period

A period of short oxygen deprivation has not been observed to induce any overt neuropathological changes in the cerebrum cerebellum or brain stem of fetal and neonatal rats at the level of the light microscope (Vannucci & Duffy 1976) However when experimental animals have been subjected to prolonged partial asphyxia chronic neuropathological changes have been observed (Myers 1972 Vannucci & Duffy 1976) Similarly preliminary data from our laboratories indicate that prolonged oxygen deprivation results in permanent changes in CA metabolism in combination with certain behaviour disturbances (Hedner et al 1979)

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l cerebral glucose consumption in the artificially lated rat influence of nitrous oxide gesia and of phenobarbital anesthesia

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INGVAR, M ABDUL-RAHMAN A. & SIESJÖ B K. Local cerebral glucose consumption in the artificially ventilated rat influence of nitrous oxide analgesia and of phenobarbital anesthesia Acta Physiol Scand 1980, 109 177–185 Received 15 Oct. 1979 ISSN 0001-6772. Laboratory for Experimental Brain Research, and Department of Neurosurgery University of Lund, S eden.

The objectives of the present study which concerns local glucose consumption ($1\text{-}CMR_{gl}$) in the rat brain as measured with the [14]C-deoxyglucose technique of Sokoloff et al (1977) were (1) to provide data for $1\text{-}CMR_{gl}$ in nitrous oxide analgesia and in phenobarbital anesthesia, allowing comparison with previous results on ygen consumption, and (2) to test recent proposal that 70% N_2O markedly reduces $1\text{-}CMR_{gl}$ in (mainly) cortical structures. Under 70% N_2O $1\text{-}CMR_{gl}$ in frontal and parietal cortex was close to 0.7 μmol g min This value is in excellent agreement with previous values for cortical" xygen consumption In phenobarbital anesthesia, $1\text{-}CMR_{gl}$ was lower than that expected from oxygen consumption, probably reflecting the fact that barbiturate anesthesia is ac companied by consumption of endogenous substrates Experiments on adrenalectomized animals that were given local anesthesia and protected from external stimuli failed to demonstrate that 70% N_2O depresses $1\text{-}CMR_{gl}$. In fact, N_2O was found to increase $1\text{-}CMR_{gl}$ in many structures. It is concluded that if $1\text{-}CMR_{gl}$ is lower in ventilated animals than in spontaneously breathing, conscious controls, the depression is more likely to be due to the neuromuscular blockade

Key ords Brain metabolism, local glucose consumption, nitrous oxide analgesia, phenobarbital anesthesia, neuromuscular blockade

evelopment by Sokoloff et al (1977) of the iographic [14]C-deoxyglucose technique for ring local cerebral glucose consumption in rain ($1\text{-}CMR_{gl}$) has provided a new means of ing cerebral metabolic rates in physiological athological situations By virtue of its resolu- he method has the unique feature of allowing urements of glucose consumption rates in dis- anatomical structures and functional units. In ny novel metabolic information has been ob- d on such diverse functions as those subserved e ual, auditory and olfactory ystems (for iew see Sokoloff et al 1977 Sokoloff 1977) on the effect of α- and β-adrenoceptor ists (Savaki et al 1978) as well as on local bolic rates accompanying focal and gen- zed epileptic seizures (Collins et al. 1976, jö & Abdul-Rahman 1979), or those following spreading cortical depression (Shinohara et al 1979) and cerebral ischemia (Levy & Duffy 1977).

I any study of cerebral metabolic rate it be comes a problem to define an appropriate control tate. Ideally such a state mimicks conditions pre vailing in normal resting subjects (or experimental animals) Many pathophysiological conditions par ticularly those involving changes in lung ventila- tion, blood pressure or body temperature require artificial ventilation. Furthermore immobilization is many times a prerequisite for appropriate sam- pling of tissue cerebral venous blood, and CSF or for control of physiological parameters. In im- mobilized animals, the choice of anesthetic or analgetic drugs is crucial. I their review article Smith & Woolman (1972) conclude that, in man 70% N_2O has mall effect on CBF and only a mild depressant effect on CMR_{O_2}. Experiments on dogs

…rylation rate of glucose and the amount of …ucose-6-phosphate formed which depends on the … concentrations of the sugars and on their relative … for the carrier mechanisms and for hexokinase … latter are known (see below) it is possible to … glucose utilization rates at steady state (which … phosphorylation rates) by measuring the time … of the arterial plasma specific activities of the two … and the deoxyglucose activity in tissue. For calcu… of glucose utilization rates with the equation de… by Sokoloff et al. (1977) certain conditions should … led. ( ) Plasma glucose concentrations should be … ly constant during the experiment. (*b*) The …lucose should occur in tracer concentrations. … extravascular tissue compartment should be …neous and all its parts should exchange directly … (*d*) Since measurements of ^{14}C-deoxyglu… activity in tissue are made by autoradiography it is …nable to distinguish between the free and phos…lated forms. Hence … sufficient period should pass … injection of ^{14}C-deoxyglucose to allow most of … form to disappear by phosphorylation or trans…

…lculation of l-CMR$_{gl}$ is made from the following …

$$C_i^*(T) \; k_1^* \; ^{-(k_2^*+k_3^*)T} \int^T C_p \, e^{(k_2^*+k_3^*)t} \, dt$$

$$\frac{V_m^* K_m}{V \; K_m^*} \left[\int^T \left(\frac{C_p}{C_p}\right) dt \quad ^{-(k_2^*+k_3^*)T} \int^T \left(\frac{C_p^*}{C_p}\right) e^{(k_2^*+k_3^*)t} \, dt \right]$$

…ds for glucose uptake rate per unit mass of tissue; … C_p^* are the arterial plasma concentrations of glu… and deoxyglucose; T is the time of decapitation after … injection, $C_i^*(T)$ is the amount of radioactivity in … of that time. k_1^* and k_2^* are the rate constants of …-mediated transport of deoxyglucose between … and tissue and between tissue and plasma, respec… k_3^* is the rate constant of phosphorylation of DOG … ratio of the tissue distribution space for ^{14}C … glucose and glucose. Φ is the fraction of glucose that … steady state is further metabolized after phosphoryla… while K_m^*, V_m^*, K_m, V are the Michaelis-Menten … and maximal phosphorylation rates for deoxy… and deoxyglucose respectively.

…pplying the equation T was 45 min and the con… were those given by Sokoloff et al. (1977).

…mination of l-CMR$_{gl}$-procedures

^{14}C-deoxyglucose (337 mCi mMol … New England …) in 0.5 ml of Krebs solution was infused over … 4 of 30 … th an infusion pump. 15 timed 100 µl … (including … blank) were taken during the 43 min …imental period. The samples were centrifuged and … as separated and frozen at … 80°C for later …mination of ^{14}C-activity and glucose concentration. … 45 min the animal … as decapitated … hereafter the … as removed and frozen in isopentane chilled to … C.

…asma glucose concentrations were determined …metrically with the hexokinase method (see Folber… et al. 1977). The ^{14}C-deoxyglucose activity was de…

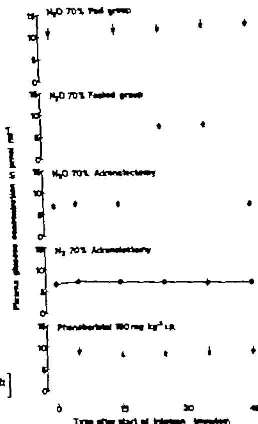


Fig. 1. The plasma glucose concentrations just before the start of deoxyglucose infusion and during the 45 min period of the expt. The values are means ± S.E.

termined by pipetting an aliquot of plasma into … scintillation vial containing 1 ml of … mixture of Soluene® and isopropanol (1 : 1 … /v). T … the vial … were then added 7.5 ml of … mixture of Instagel® and 0.1 N HCl (9 : 1 … /v). The samples were counted in … Nuclear Chicago liquid scintillation counter. The efficiency was calculated with the method of external standard ratio. The accuracy of this method was evaluated by adding an internal standard (^{14}C-hexadecane Amersham) to the vials before recounting.

Determination of ^{14}C-activity in the tissue by autoradiography was done as described previously (Abdul-Rahman et al. 1979). When recalibrating the standards it was found that the values previously assigned to them were in error by about 5%. This was due to the fact that the volume of internal standard used was sufficient to induce some quenching. Accordingly the CBF values given in that article (Abdul-Rahman et al. 1979) are about 5–10% too low.

RESULTS

The physiological parameters in the 5 experimental groups are given in Table 1. All animals had rectal

(Theye & Michenfelder 1968) and rats (Carlsson et al 1976) have given similar results indicating that 70% N_2O may provide an adequate control state in immobilized animals

So far extensive control data for l-CMR_{gl} have been published only for unanesthetized and partially restrained rats and monkeys and for rats subjected to superficial thiopental anesthesia (Sokoloff et al 1977 Sokoloff 1978 Kennedy et al 1978) Recently Sakurada Shinohara Kennedy & Sokoloff (manuscript in preparation) compared l-CMR_{gl} values obtained in spontaneously breathing rats that were either partially restrained by a plaster cast or freely moving to those measured in immobilized animals ventilated on 70% N_2O Since their results indicate that 70% N_2O depresses l-CMR_{gl} in cortical structures by up to 35% the authors question the assumption that values so obtained approximate those expected in the resting conscious state

The present experiments were undertaken to critically assess l-CMR_{gl} values obtained in ventilated rats maintained on 70% N_2O The following questions were posed (1) Are the l-CMR_{gl} values measured in reasonable agreement with those expected from CMR_{O_2} values previously obtained in immobilized rats? In order to obtain data for this comparison l-CMR_{gl} was measured in animals maintained on 70% N_2O as well as in those given phenobarbital in a dose of 150 mg kg These situations provide both "normal" and markedly reduced metabolic rates Furthermore since many experiments are conducted in fasted rats l-CMR_{gl} under 70% N_2O was assessed both in fed rats and in those fasted for 24 h (2) Does 70% N_2O depress glucose utilization in the brain? In order to answer this question l-CMR_{gl} was measured in animals maintained either on 70% N_2O–30% O_2 or on 70% N_2–30% O_2 In all of these animals pain and stressful reactions were minimized by adrenalectomy and local anesthesia

METHODS

Operative techniques and experimental groups

The experiments were performed on male Wistar rats of a SPF strain weighing 370–420 g In the majority of the animals (see below) anesthesia was induced with 3% halothane When unresponsive the animals were tracheotomized and immobilized with an i v injection of tubocurarine chloride (0.5 mg kg) Ventilation was continued with a gas mixture of 70% N_2O and 0 7% halothane in oxygen for the duration of the operation [illegible] arteries were cannulated one for recording [illegible] and the other for obtaining blood samples for [illegible] nation of blood gases pH glucose concentr[illegible] ^{14}C-deoxyglucose activity One femoral vein [illegible] lated to allow infusion of drugs and ^{14}C-de[illegible] Body temperature as measured in the rect[illegible] close to 37°C by external heating Upon com[illegible] operative procedures the halothane supply [illegible] tinued and heparin was given i v in a dose of [illegible] animal

The following 5 groups of animals were [illegible] study

1 *Fasted—70% N_2O* 6 animals were fasted [illegible] hours prior to the experiment. Following the [illegible] of operation they were maintained on 70% N_2O

2 *Fed—70% N_2O* In this and in the following [illegible] each comprising 4 animals the animals had free [illegible] pellet food until the day of operation This group [illegible] other respects similar to group 1

3 *Adrenalectomy—local anesthesia—70%* [illegible] this group the adrenal glands were removed [illegible] operation Local anesthesia (about 1 ml of 1% [illegible] per animal) was infiltrated at suture sites The [illegible] their ears plugged with cotton wool, they were [illegible] with a sterile ointment and then almost comple[illegible] bedded in cotton wool (see Carlsson et al 1976) [illegible] tion was then continued on 70% N_2O

4 *Adrenalectomy—local anesthesia—70%* [illegible] group was identical to the previous one except [illegible] that nitrous oxide supply was discontinued [illegible] before ^{14}C-deoxyglucose injection

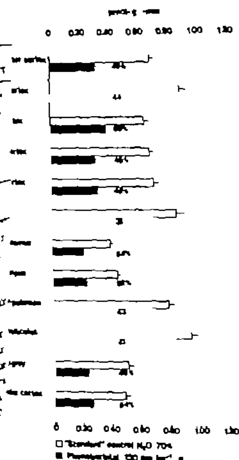
5 *Phenobarbital 150 mg/kg* This group [illegible] from the others in that anesthesia was [illegible] phenobarbital (150 mg/kg i p) However [illegible] were ventilated with 70% N_2O and 0.7% halothane [illegible] operation at the end of which ventilation [illegible] on 70% N_2 and 30% O_2

After the completion of operative procedures [illegible] mals were allowed a steady state period of 30 [illegible] the determination of l-CMR_{gl} was begun [illegible] group 4) During this time it was controlled that [illegible] in the range 35–40 mmHg and that Pa_{O_2} exceed[illegible] mmHg

Determination of l-CMR_{gl}—theoretical background

The ^{14}C-deoxyglucose technique is based on the [illegible] considerations Deoxyglucose is transported from [illegible] to cerebral intracellular fluids by the same [illegible] mechanisms as is glucose and like glucose it [illegible] phorylated by hexokinase However the [illegible] metabolism of deoxyglucose is impeded by the [illegible] is a poor substrate for glucosephosphate[illegible] (E C 5 3 1 9) and for glucose-6-phosphate dehy[illegible] (E C 1 1 1 49) Since the deoxyglucose-6-pho[illegible] (E C 3 1.3 9) activity is low in brain tissue the [illegible] essentially trapped in the tissue as deoxyglucose[illegible] phate The amount trapped at any given time [illegible] introduction in the blood then equals the integ[illegible] rate of phosphorylation by hexokinase during that [illegible] of time

A quantitative relatio[illegible]us exists betw[illegible]



The influence of phenobarbital anesthesia on local (μmol g⁻¹ min⁻¹) in the rat as compared with the 70% N_2O group. The numbers denote 1-CMR_{gl} phenobarbital anesthesia in percent of the standard

were pooled for the comparison to results ob- by Sakurada et al. In areas with high meta- rates (inferior colliculus and cerebral cortex) present values were lower than those of Saku- et al. the differences being about 30% in colliculus and 10–20% in cortical areas. ever the relative proportion of 1-CMR_{gl} be structures was very similar. In some other tures (particularly thalamus, hypothalamus, gray and cerebellar cortex to some extent caudate-putamen) the values in the two series in close agreement. In still others, a com- on could not be made since the densitometer in the present study precluded measurements very small structures.

Table 3 *Comparison of local glucose consumption (CMR_{gl}) in frontal and parietal cortex and oxygen consumption (CMR_{O_2}) in animals ventilated on 70% N_2O or given 150 mg kg of phenobarbital*

Anesthesia	CMR_{O_2} measured	CMR_{gl} calculated	CMR_{gl} found
70% N_2	4.0ᵃ	0.70	0.70
Phenobarbital 150 mg kg	2.7ᵇ	0.47	0.32

ᵃ Berntman et al. (1979)
ᵇ Nilsson & Siesjo (1975), with one aberrant value excluded (see discussion in article quoted).

2. Local CMR_{gl} I phenobarbital anesthesia

In view of the fact that the phenobarbital-anesthetized animals were paralyzed and artificially ventilated it would seem most relevant to compare the values obtained to those of the "standard" group. Fig. 2 shows the absolute values obtained in the two groups and lists the percentage reduction in CMR_{gl} induced by the barbiturate in each structure studied. In most of the structures the reduction in 1-CMR_{gl} was to about 50% of control but there were exceptions. Thus, the reduction in CMR_{gl} was somewhat less pronounced in the visual cortex and somewhat more pronounced in inferior colliculus. In the thalamus, the reduction was excessive (to a third of control).

3. A comparison between glucose and oxygen consumption rates

Previously CMR_{O_2} has been measured in animals maintained on 70% N_2O (e.g. Berntman et al. 1979) and in those given phenobarbital 150 mg kg (Nilsson & Siesjo 1975) experimental conditions being similar to the present ones. In these studies we used a modification of the Kety & Schmidt (1948) technique with sampling of cerebral venous blood from the caudal portion of the superior sagittal sinus. Assuming that this sampling site provides CMR_O values representative of mainly frontal and parietal cortex and that the CMR_{gl} values measured come close to the weighted averages for these structures we lumped together the values for these two cortical areas for comparisons. Furthermore we assumed that 95% of the glucose consumed is oxidized the remainder appearing as lactate (and pyruvate) in the venous effluent (see Hawkins et al. 1973 Norberg & Siesjo 1974). The resulting calcu-

Table 1 *Physiological parameters in experimental groups*
The values are means ± S E MABP = mean arterial blood pressure

Experimental groups	Temp (°C)	MABP (mmHg)	P (mmHg)	P_{CO_2} (mmHg)	pH
N_2O 70% fed group (n=4)	37 0±0 05	143±5	115±2	39 1±0 4	7[illegible]
N_2O 70% fasted group (n=6)	37 1±0 1	135±6	1_3±?	36 8±0 8	7[illegible]
N_2O 70% adrenalectomy + local anesthesia (n=4)	37 1±0 2	121±7	104±3	37.9+1	7[illegible]
N_2O 70% adrenalectomy + local anesthesia + N_2O (n=4)	37 3±0 1	118±6	110±3	37 4±0 8	7[illegible]
Phenobarbital 150 mg kg (n=4)	37 4±0 1	108±5	117±3	39 0±1	7[illegible]

temperatures close to 37°C Pa_O values close to or above 100 mmHg Pa_{CO} values around 40 mmHg and pH values close to 7 40 There was a tendency for blood pressure to decrease in the adrenalectomized animals ($P<0$ 05) and in those anesthetized with phenobarbital blood pressure was clearly decreased below "control" (70% N_2O $P<0$ 01)

Fig 1 shows the plasma glucose concentrations just before the start of deoxyglucose infusion and during the 45 min period of the expt In all groups except that comprising the fed animals under 70% N_2O plasma glucose concentrations were in the range 5–8 μmol g As required (see Methods) plasma glucose concentrations were [illegible] throughout the period of the expt with [illegible] tion between individual animals

Local CMR_{gl} in nitrous oxide analgesia

Table 2 lists l-CMR_{gl} values obtained in the [illegible] of animals ventilated on 70% N_2O and [illegible] them to those measured by Sakurada et al [illegible] preparation) under similar conditions ([illegible] and neuromuscular blockade) Local CMR_{gl} [illegible] differ between fed and starved animals except cerebellar cortex which had a slightly higher [illegible] the starved group ($P<0$ 05) In view of the [illegible] larity in results between fed and starved [illegible]

Table 2 *Local glucose consumption (l-CMR_{gl}) in animals ventilated on 70% N_2O with or without fast of 18–20 h as compared to data obtained by Sakurada et al (in preparation) under similar [illegible] (see text)*
The values are means ± S E

Structures	Fasted n=6	Fed n=4	Combined n=10	Sakurada et al n=5
A *Superficial cerebral structure cortex*				
Sensorimotor	0 68±0 04	0 69±0 04	0 69±0 03	0.80±0 03
Auditory	0.9 ±0 07	0 85±0 04	0 89±0 04	1 10±0 06
Visual	0 69±0 03	0 60±0 02	0 65±0 03	0.76±0 04
Parietal	0 69±0 04	0 69±0 0	0 69±0 02	0 79±0.02**
Frontal	0 67±0 03	0 76±0 03	0 71±0 01	0 87±0 02*
B *Deep cerebral structure*				
Hypothalamus	0 45±0 03	0 37±0 01	0 41±0 0_	0 43±0 04
Caudate-putamen	0 86±0 05	0 71±0 03	0 80±0 04	0 87±0 06
C *Mid-brain and Pons*				
Inferior Colliculus	1 00_0 06	0 87±0 04	0 95±0 04	1.30±0 11
Pontine gray	0.55±0 04	0 49±0 03	0 52±0 03	0.51±0 05
D *Cerebellum*				
Cerebellar cortex	0.55±0 03	0 4_±0 01	0.50±0 03	0.4 _0 03

* $P<0$ 05 ** $P<0$ 01

SSION

[...]sent results are highly pertinent to the prob[...] obtaining proper control conditions for [...] of physiological and pathophysiological [...] the brain. When discussing the results it [...]be recalled, though that such conditions [...]ry from one study to another Clearly for [...]of many physiological events it must be [...]e to compare results to those obtained in [...]hetized and spontaneously breathing ani[...]t would seem that for these the control [...]sed by Sakurada et al (in preparation see [...]okoff et al 1977) are the appropriate ones. [...]ot been excluded, though that values so [...]d are influenced by the stress of partial re[...]r of infusion and/or blood sampling in freely [...] animals (see below).

[...]ated in the introduction other studies re [...]mobilization and some form of anesthesia/ [...]m, e g that provided by 70% N_2O The [...] has remained whether or not such meas[...]arkedly alter brain metabolism and blood [...] the following, we will discuss the implica[...] the present results for these general prob[...]

[...]dity of l-CMR$_{gl}$ values in animals [...]ized on 70% N_2O

[...]gnize the difficulty of estimating a weighted [...] CMR$_{gl}$ value for any given cerebral struc[...]nd referring l-CMR$_{gl}$ and CMR$_{O_2}$ values to [...] defined tructure However since similar [...] alues were obtained if repeated den[...]ty readings were taken from different parts [...] the frontal and parietal cortices (data not [...] since alues for frontal parietal and sen[...]or cortex were similar and since the [...] sagittal sinus probably drains mainly corti[...]uctures [...]e onclude that the present l [...]alues quantitatively correspond to previ[...]surements of CMR$_{O_2}$ Since the values are [...]se to those measured with a kinetic [14]C-glu[...]chnique (Borgström et al 1976) there should [...]e doubt about the quantitative validity of the [...] obtained. True in cerebral cortical struc[...] and in the inferior colliculus, our CMR$_{gl}$ val[...]re moderately but significantly lower than [...] measured by Sakurada, Shinohara, Kennedy [...]okoff (in preparation). However the differ[...] were small enough to be explained by differ[...]ences in metabolic characteristics between the rat strains used (Sprague-Dawley versus Wistar) or by unrecognized differences in experimental conditions. Such differences may explain why Sakurada et al obtained local CBF values exceeding 2.2 ml·g min in all cortical areas except the visual cortex. To our knowledge flow rates of this magnitude have not been recorded previously

One further point should be emphasized In view of the fact that about 20% of the brain oxygen consumption is accounted for by ketone body oxidation after a 24-hour fast (Ruderman et al 1974) it may seem paradoxical that glucose consumption rates were similar in fed and fasted animals How ever since an increased proportion of glucose is metabolized to lactate (and pyruvate) in fasted animals and since arteriovenous differences in glucose concentration remain virtually unchanged (Hawkins et al 1971 Ruderman et al 1974) the present results are not surprising.

[...] Validity of l CMR$_{gl}$ values animals under phenobarbital an sthesi

The primary purpose of performing measurements on phenobarbital-anesthetized animals was to allow a comparison between CMR$_{gl}$ and CMR$_{O_2}$. At first sight, it was surprising that a good correspondence was obtained in animals on 70% N_2O but not in those anesthetized with phenobarbital. In barbiturate-anesthetized animals, the calculated CMR$_{gl}$ value (0 47 µmol·g min) even if somewhat too high (see discussion of CBF technique by Berntman et al 1979) clearly exceeds that measured with the autoradiographic technique in the appropriate cortical areas. We tentatively conclude that the differ ence is due to the fact that actual metabolic rate (i.e oxygen consumption) exceeds glucose utilization. This can be explained by the fact that barbiturates retard glycolysis at the phosphofructokinase step leading at least initially to mobilization of endogenous carbohydrate and amino acid substrates for oxidation (Chapman et al 1978) If so barbiturate anesthesia provides an example of conditions in which glucose and oxygen consumption rates are partly dissociated.

Previously the influence of thiopental on l CMR$_{gl}$ has been assessed (Sokoloff et al. 1977) Local CMR$_{gl}$ was reduced by 40-50% in areas with high metabolic rate particularly of cerebral cortical origin. Since thiopental was administered in doses that just extinguished the corneal reflex the values

Table 4 *Comparison of l CMR_{gl} in animals that were adrenalectomized and given local anesthesia and ventilated on either 70% N_2O or 70% N_2*

The values are means ± S E

Structures	70% N_2O	70% N_2
A Superficial cerebral structures cortex		
Sensorimotor	0 73±0 04	0 64±0 04
Auditory	0 97±0 05	0 72±0 03
Visual	0 81±0 05	0 63±0 02
Parietal	0 69±0 07	0 63±0 04
Frontal	0 81±0 04	0 64±0 04
B Deep cerebral structures		
Thalamus	1 05±0 05	0 67±0 03
Hypothalamus	0 48±0 02	0 41±0 03
Hippocampus	0 73±0 01	0.55±0 05
Caudate putamen	1 07±0 05	0 75±0 04
C Mid-brain and Pons		
Inferior Colliculus	0 81±0 06	0 66±0 06
Pontine gray	0 55±0 03	0 44±0 04
D Cerebellum		
Cerebellum cortex	0.5 ±0 02	0 49±0 04

$P<0.05$ $P<0.01$ $P<0.001$

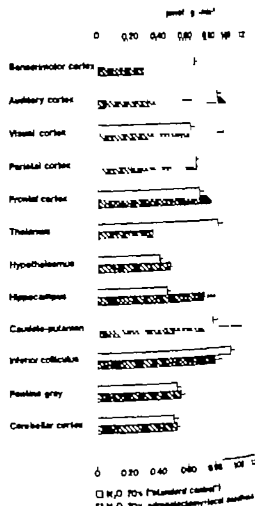


Fig 3 Local CMR_{gl} in adrenalectomized and anesthetized animals ventilated on 70% N_2O ... bars) as compared to "standard" control animals ... tained on 70% N_2O (open bars). The ... means ± S E in μmol g⁻¹ min⁻¹ $P<0.05$ ... $P<0.001$

lations (Table 3) showed an excellent agreement between expected and measured cortical CMR_{gl} in animals on 70% N O However in phenobarbital anesthetized animals the autoradiographically measured CMR_{gl} values were lower than those expected from the CMR_{O_2} measurements (see Discussion)

4 *The influence of nitrous oxide analgesia*

As stated a previous study showed no significant difference in CMR_{O_2} between animals ventilated on 70% N_2O and 70% N_2 (Carlsson et al 1976) In these stress pain and discomfort were minimized by removal of the adrenal glands by local anesthesia and by measures instituted to reduce external stimuli Using an identical preparation we measured glucose consumption rates with and without 70% N_2O (Table 4) Unexpectedly the results showed that 70% N_2O increased l-CMR_{gl} in many of the structures studied Thus although there were no or only small differences in sensorimotor and parietal cortex hypothalamus inferior colliculus pontine gray and cerebellar cortex other structures (frontal auditory and visual cortex thalamus hippocampus and caudate putamen) showed significantly increased CMR_{gl} with 70% N O results seem to exclude the possibility th... N_2O reduces metabolic rates in the brain (se... cussion)

Clearly as Fig 3 shows a complex ... emerges when the results obtained on adr... tomized nitrous oxide animal (Table 4) are ... pared to these obtained in the "standard" ... (Table 2) Thus in spite of the fact that both ... were artificially ventilated on 70% N_2O ... that were adrenalectomized injected with ... anesthetic and protected against external ... had higher l-CMR_{gl} values in frontal and ... cortex thalamus hippocampus and caudate ... men and a lower value in inferior collic... conclude that one or more of these proced... fluence local glucose c umption

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RÆDER, M MO A. AUNE, S & MATHISEN Ø Relationship between plasma pH and pancreatic HCO_3^- secretion at different intravenous secretin infusion rates. Acta Physiol Scand 1980, 109: 187–191 Received 19 Oct. 1979 ISSN 0001-6772. Institute for Experimental Medical Research, University of Oslo and Surgical Dept. 2, Ullevål Hospital, Oslo

The relationship between pancreatic HCO_3^- secretion and plasma pH during acute systemic acid-base changes was investigated in 6 anesthetized, artificially ventilated pigs (20–25 kg) at 2 different, secretin infusion rates. At 0.45 C.U./kg b wt. h secretin infusion and plasma pH 7.40±0.01 pancreatic HCO_3^- secretion averaged 61±12 μmol/min. Stepwise lowering of plasma pH through I. infusion of HCl and CO_2 administration to inspired air proportionately reduced secretion rate estimated zero HCO_3^- secretion occurring at plasma pH 7.01 Subsequent I. secretin infusion at 2 70 C.U./kg b wt. h^{-1} increased HCO_3 secretion to 249±42 μmol/min at plasma pH 7.33+0 04 stepwise lowering of plasma pH proportionately reduced HCO_3^- secretion to estimated zero at plasma pH 6.71 A reduction of plasma pH by 0 1 pH unit reduced HCO_3^- secretion during low and high rate of secretin infusion by 18±3 μmol/min and 35±8 μmol/min, respectively Secretin infusion rate did not affect pancreatic chloride excretion. These findings support the view that secretin increases HCO_3^- secretion, and hence proton transport to the interstitial fluid, by augmenting the proton motive force developed by HCO_3^- secreting cells.

Key words Acid-base hypothesis, pig, proton motive force proton pump

...atic HCO secretion implies equimolar ...ort of protons to the interstitial fluid by secreting cells (Davies 1949) It has recently demonstrated that plasma pH determines the ...rat of pancreatic HCO_3^- secretion. during ...int secretin infusion stepwise lowering of ...a pH proportionately reduces HCO_3^- secre...tion and secretion is abolished at plasma pH 6 90 ...et al. 1979).

...retin is a major stimulant of pancreatic HCO_3^- ...tion but it is not clear how the relationship ...een plasma pH and pancreatic HCO_3^- secretion ...with secretin infusion rate One possibility ...d be that secretin increases HCO_3^- secretion as ...s lowers the plasma pH value at which HCO_3^- ...tion is abolished by acidosis. This would har...re with the view that secretin increases HCO_3 ...tion through raising the proton motive force by HCO^- secreting cells. The other possibility would be that HCO_3^- secretion would increase upon augmenting secretin infusion rate at any plasma pH exceeding 6.90: at plasma pH<6.90 the mechanism responsible for HCO^- secretion might be refractory to secretin stimulation This would be in accordance with the view that acidosis reduces HCO_3^- secretion through inhibiting the metabolism in HCO_3^- secreting cells, near complete metabolic inhibition occurring at plasma pH 6.90.

The purpose of the present study was to distinguish between these alternatives. The relationship between plasma pH and pancreatic HCO_3^- secretion was studied in 6 anesthetized pigs during acute systemic acid-base disturbances at two different rates of I secretin infusion We found that increasing i.v secretin infusion from 0 45 to 2.70 C.U./kg b wt. h^{-1} increased both the rate of pancreatic HCO_3^- secretion and lowered the plasma pH limit at which HCO_3^- secretion is abolished by

...in plasma pH (correlation coefficient, ... until HCO_3^- secretion ultimately ceased ... At secretin infusion rate of 0.45 C U./kg ... lowering of plasma pH by 0.1 unit re... pancreatic HCO_3 output by an average of ...mol/min, estimated zero secretion occurred ...ma pH 7.01. The inhibition of HCO_3^- secre... low plasma pH was reversible as shown by ...mediate return of HCO_3^- secretion upon nor...tion of plasma pH through withdrawal of CO_2 ...ne inspired air/O_2 mixture and further back...n of plasma pH with i.v. $NaHCO_3$ infusion.

...n secretin infusion rate was increased to 2.70 ...g b.wt. h^{-1} pancreatic HCO_3 secretion rose ... 42 µmol/min at plasma pH 7.33±0.04 and ...gnificantly higher ($P<0.05$) than during 0.45 ...g b.wt. h secretin infusion rate at all plas... values between 7.60 and 6.76. During step... lowering of plasma pH at 2.70 C U./kg b.wt ...retin infusion rate, pancreatic HCO_3 secre...ell in proportion to changes in plasma pH ...0); a reduction in plasma pH by 0.1 unit ... these circumstances reduced pancreatic ... output by 35±8 µmol/min, which is signifi... more than the reductions in HCO_3^- secretion ...ved during lowering of plasma pH at 0.45 ...kg b.wt. h secretin infusion rate. Estimated ...secretion occurred at plasma pH 6.71 during ...U./kg b.wt h secretin infusion rate. Thus, ...lation to augmenting pancreatic HCO_3^- output, ...crease in secretin infusion *rate shifted* the ...ma pH limit at which HCO_3^- secretion was ...shed.

...oride secretion was 14±1 µmol/min at plasma ...39±0.01 during low secretin infusion rate and ...µmol/min at plasma pH 7.33±0.04 during high ...ecretin infusion rate. The difference is not ...icant, thus secretin had no certain effect on ...reatic chloride transport under these cir...stances (Table 1).

...CUSSION

...present study demonstrates a linear relation... between plasma pH and pancreatic HCO^- sec... at both high and low rates of i.v. infusion of ...retin. Increasing secretin infusion rate from 0.45 ...70 C U./kg b.wt. h raised pancreatic HCO_3^- ...ut and lowered the plasma pH limit at which ...O secretion was abolished by acidosis from pH ...to pH 6.71. Furthermore, the reduction in HCO secretion elicited by a decimal unit reduction of plasma pH was greater during high than low rate of i.v. secretin infusion.

Recently we suggested that a proton pump in the interstitial cell membrane of HCO_3 secreting cells may be responsible for proton transport to the interstitial fluid (Rieder et al. 1979). Changes in plasma pH proportionately alters HCO_3 secretion rate while changes in pancreatic juice pH elicited by altering arterial P_{CO_2} are without effect on secretion rate; thus, HCO_3^- secretion is affected by pH changes only at the interstitial cell border of HCO_3 secreting cells (Rieder et al. 1979). The reason for this assymetric sensitivity of HCO_3^- secreting cells to external pH is not clear. One explanation could be that reductions in interstitial fluid pH could increase back leakage of protons across the interstitial cell membrane and thus reduce net transport of protons from HCO_3 secreting cells. Alternatively reductions in interstitial fluid pH could increase the electrochemical proton potential of interstitial fluid and curtail the proton potential difference between HCO_3^- secreting cells and interstitial fluid. Accordingly secretion would cease when the proton potential difference over the interstitial cell membrane was zero. Finally interstitial fluid proton concentration could inhibit metabolism in HCO_3 secreting cells: changes in metabolic rate and hence energy supply to the proton transport mechanism, could cause proportionality between the rate of proton transport and plasma pH.

The effects of secretin observed in the present study harmonize with the view that secretin increases the proton motive force generated by HCO_3^- secreting cells. An increase in proton motive force would both augment proton transport rate and sustain proton transport at higher interstitial fluid proton concentrations. We found greater increments in HCO_3^- secretion per decimal pH unit increase in plasma pH at high secretin infusion rate: this would indicate that the proton motive force increases more with plasma pH under these conditions.

Augmenting secretin infusion rate restored HCO secretion at plasma pH <7.01. This indicates that acidosis does not impair proton transport through reducing metabolism in pancreatic cells. If metabolism were suppressed to the point that it could no longer sustain proton transport at plasma pH 7.01, increasing secretin infusion rate from 0.45 to 2.70 C U./kg b.wt. h would not be expected to yield any secretory response from the pancreas at

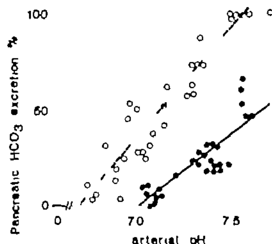


Fig 1 Relationship between pancreatic HCO_3 excretion and plasma pH during acute acid-base changes in anesthetized secretin infused pigs. Plasma pH was varied by infusion of HCl intravenously and addition of CO_2 to the respired air/O_2 mixture 100% = maximal secretory rate obtained in each experiment. ● = 0 45 C U secretin/kg b wt. h i v O = 2.70 C U secretin/kg b wt h i v Oblique lines represent calculated linear regression for relationship between HCO_3^- excretion and arterial pH at the two different secretin infusion rates Each animal served as it own control Number of expts. 6.

acidosis from pH 7 01 to pH 6 71 A reduction in plasma pH by 0 1 pH unit reduced HCO_3 secretion more at high i v secretin infusion rate than at low infusion rate These findings favour the view that secretin may increase pancreatic HCO secretion through an effect on the force driving protons from HCO_3 secreting cells to the interstitial fluid

METHODS

The experiments were carried out on 6 young pigs (20–25 kg) during general pentothal anesthesia (40 mg/kg b wt.) additional pentothal was given i v as required during the expts Anesthesia was supplemented by periodic administration of pancuronium bromide (Pavulon) The animals were artificially ventilated an endo-tracheal tube ensured free airways All animals were killed upon completion of the expts

Blood pressure was monitored by a Statham transducer (P23Gb) via a polythylene catheter inserted into one carotid Vagal nerves were left intact Both jugular veins were cannulated one for secretin infusion the other for electrolyte infusions

The abdomen was opened by a midline incision For timed collection of pancreatic juice the main pancreatic duct was cannulated by a soft polythylene catheter through a duodenotomy incision. The gallbladder and urinary bladder were drained by indwelling catheters The common bile duct and pylorus were occluded [illegible] tures During the operation 900 ml [illegible] fused i v Following surgery the abdomen [illegible] with clips. The body temperature of the [illegible] constant by an electric pillow

Secretin (from the Karolinska Institute, [illegible] infused i v at a steady rate of 0.45 C.U/kg [illegible] the initial half of the expts subsequently [illegible] of 30 C.U secretin was injected i.v [illegible] infusion continued at 2 70 C U/kg b.wt. h

Upon reaching steady state after commencing [illegible] infusion paired 4 min samples of pancreatic [illegible] obtained Arterial blood specimens were [illegible] middle of each collecting period for electrolyte [illegible] and immediate pH and P_{CO_2} determination [illegible] timed collection of pancreatic juice 300 µl [illegible] juice were sampled on capillary tubes for [illegible] and P_{CO_2} determination

Plasma pH was lowered from control by [illegible] ministration of HCl i v up to a total of [illegible] solved in 300–500 ml water further lowering of [illegible] was achieved through stepwise increase of P_{CO_2} [illegible] air/oxygen mixture

At each step during steady state 4 min [illegible] pancreatic juice and blood specimens were [illegible] analysis Arterial pH was subsequently restored [illegible] malization of P_{CO_2} in inspired air mixture and [illegible] titration of plasma pH with 150–160 mmol [illegible] 900–1 000 ml water with 5 mmol KCl added The [illegible] dure for lowering plasma pH in steps was [illegible] secretin infusion rate of 2.70 C U/kg b wt. h [illegible] collections of pancreatic juice and blood [illegible] each step

Pancreatic juice plasma pH and P_{CO_2} [illegible] an Instrumentation Laboratories Blood Gas [illegible] (Lexington Mass. U S.A) and plasma HCO_3^- [illegible] tion was calculated according to Henderson [illegible] equation. Pancreatic juice HCO concentration [illegible] mated as the difference between the sum of Na [illegible] concentrations and Cl^- concentration in pancreatic [illegible] Na and K concentrations in plasma and pancreatic [illegible] were measured by an Instrumentation Laboratories [illegible] flame photometer Plasma and pancreatic juice [illegible] tions were made with a CM 10 chloride titrator [illegible] ter Copenhagen Denmark)

Statistical methods

Wilcoxon s rank test for unpaired measurements [illegible] Whitney test) was used to evaluate differences [illegible] observations (Snedecor 1967) Linear regression [illegible] calculated using the method of least squares fit

RESULTS

At control plasma pH 7 40±0 01 pancreatic [illegible] secretion averaged 78±9 µmol/min during [illegible] sion of secretin at 0 45 C U./kg b w t h [illegible] lowering of plasma pH through i v infusion [illegible] and addition of CO to the inspired air/O_2 [illegible] reduced HCO secre [illegible] roportion to the [illegible]

	HCO₃⁻ (μmol/min)	Cl⁻ (μmol/min)
	78 ±9	13 ±1
	249 ±42	19 ±5

...however the positive relationship of plasma ... concentration to HCO_3^- secretion which is ... eported (Case et al 1970 Pak et al 1966 ... 1972) may not be causative as different ...nships pertain between HCO_3^- secretion and ... HCO_3^- concentration at normal and high ... a contrast, there is an unequivocal relation... etween plasma pH and HCO_3^- secretion under ... conditions (Ræder et al. 1979) Plasma pH ... ore may be the relevant ionic determinant of ... ate of HCO_3 secretion during acid-base ... es. Furthermore trebling of pancreatic juice ... concentration as during high P_{CO_2} is with... ffect on the rate of secretion (Ræder et al ... the proposed HCO_3-ATPase mechanism ...sts that an increase in proton supply at the ...al cell border enhances proton transport ... observations therefore, argue against the va... of holding luminal membrane bound HCO_3-... are responsible for pancreatic HCO^- secre...

...e results of the present study therefore may ... be interpreted within the framework of pan... ic HCO_3 secretion being due to a pancreatic ... proton pump orientated towards the interstitial ... and responding quantitatively to secretin ... ulation by increasing the proton motive force

This study was supported by the Norwegian Research Council for Science and the Humanities Professor Carl Semb Fund for Medical and Surgical Research, Doctor Alexander Malthe's Legacy and Ullevaal Hospital Gastroenterological Research Fund. The experiments were carried out at the Institute of Experimental Medical Research, University of Oslo. The skilled assistance of Miss Aase Kristin Andresen, Miss Inger Bjerkodal Mrs Patricia Cooper Mrs Anna Croucher Mrs Ellen Dahl, Miss Nina Eriksen Mr Erik Faestelien Mr Bjørn Kristiansen Mr Severin Larsand B.Sc Mrs Grethe Larum, Mr Ove Moen and Mrs Åshild Salvesen is gratefully acknowledged.

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Table 1 *Pancreatic juice composition and secretion at different intravenous secretin infusion rates*

	Arterial concentrations			Pancreatic juice concentrations					
Secretin infusion rate	pH	P_{CO_2} (kPa)	HCO_3 (mmol/l)	pH	P_{CO_2} (kPa)	HCO (mmol/l)	Na (mmol/l)	K (mmol/l)	Cl
0 45 C U /kg b wt h	7 40 ±0 01	5.37 ±0.26	22.9 ±0 4	8 14 ±0 02	4 79 ±0.20	133 ±5	154 ±1	4 4 ±0 2	[illegible]
2 70 C U./kg b wt. h	7.33 ±0 04	6 48 ±0 03	24 8 ±2 1	8 16 ±0 02	5 14 ±0.39	144 ±4	151 ±1	4 5 ±0 4	[illegible]

Data presented are mean values ± S E from 6 expts on anesthetized young pigs (20–25 kg) each animal serving as control

plasma pH <7 01 Also the immediate return of HCO_3 secretion and proton transport upon normalization of plasma pH after acidosis argues against extensive interference with metabolism in HCO secreting cells

Secretin could increase HCO_3 secretion through reducing back-leakage of protons from the interstitial fluid into HCO_3 secreting cells This could be the case if secretin reduced interstitial cell membrane proton permeability However the concept of proton back-leakage as regulator of net proton transport during acute acid-base changes implies that proton back-leakage would increase with increasing interstitial fluid proton concentration The inhibition of HCO_3 secretion elicited by decimal unit reduction of plasma pH increased in the present study when secretion infusion rate was raised this would indicate more rather than less proton back leakage under these circumstances Therefore the possibility that secretin may increase HCO_3 secretion through reducing proton permeability of the interstitial cell membrane of HCO_3 secreting cells is viewed as unlikely

So far the results of our study have been discussed with reference to the proton pump hypothesis However other possibilities should be considered It has been proposed that pancreatic proton flux is secondary to transcellular Na transport (Swanson & Solomon 1972) According to this hypothesis two Na-H ion exchange mechanisms are present in pancreatic cells—one in the interstitial cell membrane the other in the luminal cell membrane of HCO_3 secreting cells The former pump would be driven by the electrochemical potential for Na movement into cells the latter pump would be linked directly to metabolism (Swanson & Solomon 1975) The energy available for proton secretion from pancreatic cells would be proportional to the logarithm of the ratio between Na concentrations outside and inside secretory cells, [illegible] one to-one ion exchange ratio under these [illegible] tions the ion exchange would be independent [illegible] cell membrane potential (Swanson & So[illegible] 1975)

To account for how secretin may increase [illegible] force driving protons to the interstitial fluid [illegible] Na H ion exchange mechanism one may [illegible] tulate that secretin would lower intracellular [illegible] concentrations in HCO_3 secreting cells. this [illegible] increase the potential energy available for Na-H exchange at the interstitial cell membrane [illegible] ever it has been reported that cell Na con[illegible] tion increases with the onset of HCO_3 secr[illegible] (Scratcherd & Case 1973) Alternatively [illegible] crease in driving force would result if the Na-H exchange ratio was increased by secretin, i e [illegible] Na ions moving into the cell per proton se[illegible] as secretin infusion rate was augmented [illegible] would either establish an Na carousel and [illegible] Na K ATPase at the interstitial cell border [illegible] to increased transcellular transport of NaCl [illegible] present study we could find no evidence of [illegible] increasing pancreatic transport of NaCl.

It has also been proposed that pancreatic [illegible] secretion is mediated by HCO_3-ATPase loc[illegible] the luminal cell membrane According to [illegible] scheme protons would be actively transp[illegible] HCO_3-ATPase from duct lumen across the lu[illegible] cell membrane into cells where they would be [illegible] fered by HCO_3 ions diffusing into the cells fro[illegible] interstitial fluid The ensuing carbonic acid [illegible] dehydrate to CO_2 which would diffuse the lumen where it would rehydrate ionize and [illegible] HCO_3 ions for excretion as well as carbo[illegible] OH ions with formation of HCO ions the pr[illegible] would recycle (Schultz et al 1971) Secretion [illegible] be proportional to HCO delivery into sec[illegible] cells—i e proportional [illegible] l sma HCO conc[illegible]

ıtant flow vs constant pressure-perfusion tudies of pulmonary vasoactive responses

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BJERTNÆS L. & HAUGE, A Constant flow- vs. constant pressure-perfusion for studies of pulmonary vasoactive responses. Acta Physiol Scand 1980 109 193–200 Received 23 Oct. 1979 ISSN 0001-6772. Institute of Physiology University of Oslo Norway

W have compared the pulmonary vascular responses to standardized hypoxic vasoconstrictor stimulus (F_{IO_2} = 0 02) obtained during 1) constant volume inflow with pulmonary arterial pressure as the dependent variable, and 2) constant inflow pressure, with flow as the dependent variable. Isolated rat lungs were perfused at different baseline transvascular pressures The xperimental arrangement allowed changes between the tw types of perfusion. Hypoxia at constant pressure perfusion gav higher percentage rise in pulmonary vascular resistance (PVR) at all pressure levels. This advantage was however more than offset by the finding that) vascular closure (total or partial) often occurred, particularly below arterial pressure of 3 kPa, making detection of graded responses impossible and b) the control situation was rarely regained. Responses obtained during constant flow were less reduced by elevations in baseline transvascular pressure and the control situation was rapidly and completely regained The observation that hypoxic ascular closure may occur in the pulmonary vascular bed supports the hypothesis that high altitude edema is caused by precapillary occlusion of major part of the vascular bed, thereby subjecting still perfused regions to very high pressures and flow

Key ords Lung vessels, vascular smooth muscle arteriolar wall stress transmural pressure, hypoxia, critical closure

s work e have explored the relative merits of ant flow and constant pressure perfusion sys- for the ssessment of a vasoconstrictor re se in the pulmonary circulation As the lardized stimulus for vasoconstriction, we used a hypoxic ventilation gas mixture =0 02). In order to quantitate the pulmonary ular response to acute alveolar hypoxia and the ge in the response caused by g anesthetic ts we have previously used a constant flow usion technique (Hauge 1968 Bjertnæs 1977) A ge in ascular resistance caused by hypoxia-ced vasoconstriction was measured as a rise in lonary arterial pressure This commonly used nique for studies of n vasoconstrictor-stimuli recently been criticized (Benumof & Wahrenk 1975) It was pointed out that the induced rise ntravascular pressure will attenuat the re nse making the test-system rapidly les sensi as the constrictor-stimulus is increased A con pressure system with flow as the dependent variable should consequently be a better technique for evaluation of pulmonary vascular responses

The degree of attenuation of the vasoconstrictor response during constant flow perfusion will de pend on several factors such as the radius of the essels involved and the level of initial circumferential smooth muscle stretch. Gore (1977) has investigated the relationsh p between magnitude of active constriction and initial stress in the arterioles of the frog mesentery but this relationship is not known for the small pulmonary arteries involved in the response to hypoxia.

Because there is no general agreement on the advantages of constant pressure as compared with constant flow perfusion we decided to compare the vasoconstrictor responses obtained at the two modes of perfusion at different intravascular pressure levels. In addition we have discussed the significance of our findings in relation to high altitude pulmonary edema and the phenomenon of critical closure of essels.

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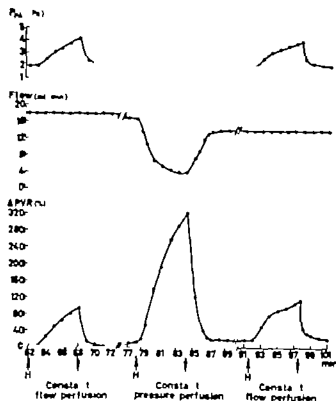


Fig. 1. Recordings of pulmonary arterial pressure (P), blood flow and calculated changes in pulmonary vascular resistance (ΔPVR). Three 6 min periods of ventilation hypoxia are indicated by arrows (H). The first and the third response to hypoxia is obtained during constant flow, the middle during constant pressure perfusion. Base line (control) arterial pressure was the same. A slight flow reduction, also reflected as a small increase in PVR, occurred on changing from constant flow to constant pressure perfusion.

The hypoxic stimulus resulted in the largest response expressed as ΔPVR, during constant pressure perfusion, but baseline flow and PVR were not regained.

METHODS

Operative technique

Rats of the Wistar strain, weighing between 240 and 360 g were exposed to diethyl ether until they became unconscious; pentobarbital 3–4 mg/100 g was then injected intraperitoneally.

A tracheostomy was performed and continuous positive pressure ventilation started. The chest was opened through a median sternal approach. The trachea, the lungs and the main vessels were dissected free from adjacent tissue. The pericardium was opened. The esophagus was ligated just above the diaphragm and cut. A loose ligature was tied around the inferior caval vein above the diaphragm and a second one around the pulmonary artery, aorta and the superior caval vein just above heart level. Half a ml containing 50 I.U. pure powdered heparin (Novo) in isotonic saline was slowly injected into the left ventricle. The ligature around the inferior caval vein was tightened and the ventilation stopped. The ... preparation was then removed from the chest. A ... steel cannula fitted with two extra inlets, one for ... monitoring and one for constant pressure per... inserted into the pulmonary artery and the ligature tightened. Similarly a larger stainless steel cannula was inserted through the left ventricle into the l... Both cannulae and the silicone rubber tubing of the ... ing system were provided with luer male/female ... tions for rapid coupling, thereby reducing the ... out pulmonary blood flow. The time which ... tween arrest of the animal's own circulation and ... perfusion was between 5 and 13 min (mean ...

The lung preparation with cannulae was ... humidified perspex chamber surrounded by a ... stated water jacket, where it hung freely suspended ... inflow cannula.

Perfusion

The vascular bed was perfused via the arterial ... constant volume inflow or constant inflow pre... experiment was always started with constant f... sion using a roller pump (LKB, Sweden). The effluent was drained into a thermostated perspex ... From a bottom outlet the perfusate was pumped ... the pulmonary artery to the lungs, and then ... through the left atrial cannula to the top inlet of ... voir. This was covered by a piece of "paraf... sected only by a hole for the venous tubing. ... monary arterial pressure and flow was measured ... Statham P 23Db pressure transducer and ... transducer connected to a Sanborn polygraph. ... was also measured by collecting effluent perfusate ... graded clyinder for standardized periods of ... few experiments left atrial pressure elevation ... carried out by leading the effluent blood ... the desired connection in a vertical ladder of ... and silicon rubber tubing. Stepwise elevation of ... pressure was obtained by closing the appropriate ... tube connections for the time periods required.

Constant inflow pressure perfusion was established ... pumping the blood from the venous reservoir ... ladder of perspex and silicon rubber tubing ... the arterial side. Inflow pressure could be set at ... level by clamping the rubber tube connections ... desired level above the lungs. From this level ... flowed into the arterial cannula. Any excess ... ered by the pump and not accepted by the ... vascular bed under the prevailing pressure ... drained back into the blood reservoir. In ... drainage pathway was clamped. Constant ... pressure during a vasoconstrictor response was ... tained by manual reduction in pump output. The ... of this arrangement was to maintain the pulse ... amplitude also at the lowest flow levels, during ... We found that by this arrangement the mean ... sure could be kept constant within ±0.13 kPa ... flow reduction. Mean and undamped pulmonary ... pressure was measured simultaneously.

Ventilation

After one or two gentle inflations of the lungs ... pressure ... ventilation was started using a Starling ...

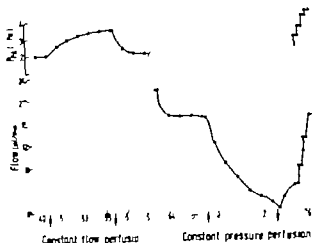


Fig. 2. Recordings of pulmonary arterial pressure (P_{PA}) and flow. Vasoconstrictor responses to hypoxia (between arrows) were evoked, first during constant flow perfusion, then during constant pressure perfusion, starting from the same baseline (control) arterial pressure. A spontaneous flow reduction occurred when perfusion was changed from one of constant flow to one of constant pressure. During the subsequent hypoxic period flow fell to zero. Control flow level was only regained after stepwise increases in arterial pressure to twice the initial level. Abscissa is time after start of lung perfusion.

pump (C. F. Palmer Ltd.). Tidal volume was ... and 2 ml (constant in each expt.), giving ... peak ... sure of about 1 kPa at start of perfusion and ... The ventilation frequency was fixed at 80 ... r min. End expiratory pressure ... as kept constant 0.2 kPa by means of a water seal. Tracheal ... was recorded by ... differential pressure transducer 270 Hewlett Packard), connected to the recorder. The lungs were ventilated with ... gas consisting of 21% oxygen, 5% carbon dioxide ... nitrogen. Airway hypoxia was created by ventilating the lungs with ... gas mixture containing 2% ... % carbon dioxide and 93% nitrogen. The preparation experimental model are modifications of those ... by Hauge (1968 ... b).

... 0 ml blood from 3 to 5 unanesthetized donor rats, ... ined by heart puncture. To each 10 ml of blood ... ed 10 I.U. of heparin (Novo). Air was evacuated ... perfusion system (tubings and connections) by ... Krebs-Ringer solution through it. Just prior to ... of lung perfusion, the perfusate blood (which had ... ted for about 1 h in ... closed perspex bottle at 778° ... was substituted for the Krebs-Ringer solution ... the pH ... as controlled by using ... Radiometer acid-... lyzer (PHM71).

...LTS

... entilation hypoxia elicited a vasoconstric... ponse a few seconds after the entrance of the ... ic gas mixture (F_{IO_2} = 0.02) into the alveoli.

Fig. 1 gives examples of responses obtained during constant flow and constant pressure perfusion in one pair of lungs. The period of hypoxia was 6 min each time and the vasoconstriction was measured either as a rise in pulmonary arterial pressure or as a reduction in flow. When the response is expressed as a change in pulmonary vascular resistance (ΔPVR) the standardized vasoconstrictor stimulus used in this example caused a response which was 3.5 times larger during constant pressure perfusion than during perfusion with constant flow.

Fig. 2 gives another example in which the hypoxic period was 6 min during both modes of perfusion. First, a response to hypoxia obtained during constant flow perfusion was detected as a reversible rise in pulmonary arterial pressure. When a constant pressure perfusion was established, flow was transiently reduced before the correct inflow pressure was obtained. This purely passive mechanical type of event resulted in ... lasting PVR increase as reflected in the new lower flow. When the hypoxic stimulus was introduced an unstable situation was created. The vasoconstriction resulted in a zero flow condition in spite of unaltered pulmonary arterial pressure. When the hypoxic stimuli ... was withdrawn only a modest increase in flow occurred. In order to regain flow it was necessary to increase inflow pressure. Thus it appeared

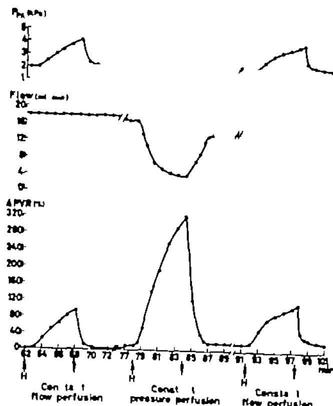


Fig 1 Recordings of pulmonary arterial pressure (P_A) blood flow and calculated changes in pulmonary vascular resistance (ΔPVR) Three 6 min periods of ventilation hypoxia are indicated by arrows (H) The first and the third response to hypoxia is obtained during constant flow the middle during constant pressure perfusion. Base line (control) arterial pressure was the same A slight flow reduction also reflected as a small increase in PVR occurred on changing from constant flow to constant pressure perfusion

The hypoxic stimulus resulted in the largest response expressed as ΔPVR during constant pressure perfusion but baseline flow and PVR were not regained

METHODS

Operath techniqu

Rats of the Wistar strain weighing between 240 and 760 g were exposed to diethyl ether until they became unconscious pentobarbital 3–4 mg/100 g wa then injected intraperitoneally

A tracheostomy was performed and continuous posit e pressure ventilation started The chest was opened through a median sternal approach The trachea the lungs and the main vessels were dissected free from adjacent tis ue The pericardium was opened The esophagus wa ligated just above the diaphragm and cut A loose ligature was tied around the inferior caval vein above the diaphragm and a second one around the pulmonary artery aorta and the superior caval vein just above heart level Half a ml containing 100 I U pure powdered heparin (Novo) in isotonic saline was slowly injected into the left ventricle The ligature around the inferior caval vein was tightened and the ventilation stopped The [illegible] preparation was then removed from the chest [illegible] steel cannula fitted with two extra inlets, one for [illegible] monitoring and one for constant pressure [illegible] inserted into the pulmonary artery and the [illegible] ened Similarly a larger stainless steel [illegible] was inserted through the left ventricle into the [illegible] Both cannulae and the silicone rubber tubing of the [illegible] ing system were provided with luer male [illegible] tions for rapid coupling thereby reducing the [illegible] out pulmonary blood flow The time which [illegible] tween arrest of the animal's own circulation [illegible] perfusion was between 5 and 13 min (mean [illegible]

The lung preparation with cannulae was [illegible] humidified perspex chamber surrounded by a [illegible] stated water-jacket where it hung freely [illegible] inflow cannula.

Perfusion

The vascular bed was perfused via the arterial [illegible] constant volume inflow or constant inflow [illegible] experiment was always started with constant [illegible] sion using a roller pump (LKB Sweden) The effluent was drained into a thermostated perspex [illegible] From a bottom outlet the perfusate was pumped [illegible] the pulmonary artery to the lungs, and then [illegible] through the left atrial cannula to the top inlet of [illegible] voir This was covered by a piece of [illegible] sected only by a hole for the venous tubing [illegible] monary arterial pressure and flow was measured [illegible] Statham P 23Db pressure transducer and [illegible] transducer connected to a Sanborn polygraph [illegible] was also measured by collecting effluent [illegible] graded clylinder for standardized periods of [illegible] few experiments left atrial pressure elevation [illegible] carried out by leading the effluent bloodstream [illegible] the desired connection in a vertical ladder of [illegible] and silicon rubber tubing. Stepwise elevation of [illegible] pressure was obtained by closing the appropriate tube connections for the time periods required

Con t nt infl w pres ure perfusion was est [illegible] pumping the blood from the venous reservoir [illegible] ladder of perspex and silicon rubber tubing [illegible] the arterial side Inflow pressure could be set [illegible] level by clamping the rubber tube connection [illegible] desired level above the lungs From this level [illegible] flowed into the arterial cannula Any excess [illegible] ered by the pump and not accepted by the [illegible] vascular bed under the prevailing pressure [illegible] drained back into the blood reservoir In 2 [illegible] drainage pathway was clamped Constant mean [illegible] pressure during a vasoconstrictor response [illegible] tained by manual reductions in pump output. The [illegible] of this arrangement was t maintain the pulse [illegible] amplitude also at the lowest flow-levels, during [illegible] We found that by this arrangement the mean [illegible] sure could be kept constant within ±0 13 kPa [illegible] flow reductions. Mean and undamped pulmonary pre sure was measured simultaneously

V ntilati n

After one or two gentle inflations of the lungs [illegible] pressure *ntil tion* was started using a Starling

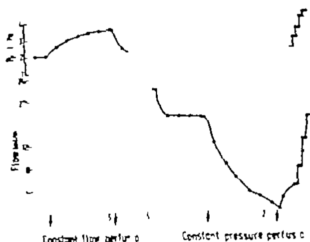


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on pump (C. F. Palmer Ltd.). Tidal volume was 1 and 2 ml (constant in each xpt.), giving peak pressure of about 1 kPa at start of perfusion and ion. The ventilation frequency was fixed at 80 per min. End expiratory pressure as kept constant about 0.2 kPa by means of water seal. Tracheal as recorded by differential pressure transducer Model 270 Hewlett-Packard), connected to the recorder. The lungs were ventilated with gas consisting of 21% oxygen, 5% carbon dioxide and % nitrogen. Airway hypoxia was created by ventilating the lungs with gas mixture containing % 5% carbon dioxide and 93% nitrogen. The preparation and experimental model are modifications of those ed by Hauge (1968a, b).

Perfusate

—40 ml blood from 3 to 5 anesthetized donor ats obtained by heart puncture. T each 10 ml of blood was 50 I.U. of heparin (Novo). Air was evacuated from the perfusion ystem (tubings and connections) by ing Krebs-Ringer solution through it. Just prior to start of lung perfusion, the perfusate blood (which had stored for about 1 h in closed perspex bottle at 77°) was substituted for the Krebs-Ringer solution. The pH as controlled by using Radiometer acid-base analyzer (PHM71).

...ULTS

...entilation hypoxia elicited vasoconstrictor response few seconds after the entrance of the hypoxic gas mixture (F_{IO_2}=0.0...) into the alveoli.

Fig. 1 gives examples of responses obtained during constant flow and constant pressure perfusion in one pair of lungs. The period of hypoxia was 6 min each time, and the vasoconstriction was measured either as a rise in pulmonary arterial pressure or as a reduction in flow. When the response is expressed as a change in pulmonary vascular resistance (ΔPVR) the standardized vasoconstrictor stimulus used in this example caused a response which was 3.5 times larger during constant pressure perfusion than during perfusion with constant flow.

Fig. 2 gives another example in which the hypoxic period was 6 min during both modes of perfusion. First, a response to hypoxia obtained during constant flow perfusion was detected as a reversible rise in pulmonary arterial pressure. When a constant pressure perfusion was established flow was transiently reduced before the correct inflow pressure was obtained. This purely passive, mechanical type of event resulted in a lasting PVR increase as reflected in the new lower flow. When the hypoxic stimulus was introduced an unstable situation was created. The vasoconstriction resulted in a zero flow condition in spite of unaltered pulmonary arterial pressure. When the hypoxic stimulus was withdrawn only a modest increase in flow occurred. In order to regain flow it was necessary to increase inflow pressure. Thus, it appeared

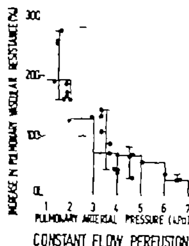


Fig 3 Percentual increases in pulmonary vascular resistance caused by standardized periods of ventilation hypoxia in a group of rat lungs perfused with constant flow Each point represents one response The columns and bars indicate the median increase in vascular resistance and range of responses respectively for each 1 kPa rise in baseline (control) pulmonary arterial pressure

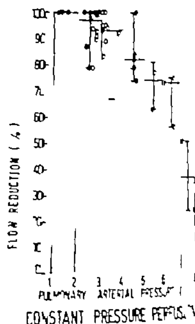


Fig 4 Percentual pulmonary flow reduction in standardized periods of ventilation hypoxia in rat lungs perfused with constant inflow pressure at various levels of pulmonary arterial pressure point represents one response The columns indicate the median flow reduction and range of respectively for each 1 kPa rise in baseline arterial pressure

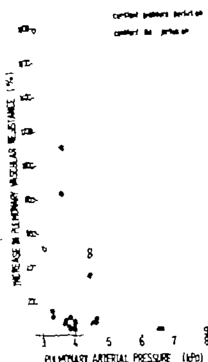
as if a part of the vascular bed remained permanently closed

The two above experiments are typical for the differences encountered when a standardized vasoconstrictor stimulus was introduced during the two modes of perfusion Constant pressure perfusion gave a higher percentage rise in PVR than constant flow perfusion This apparent advantage for quantitation of a vasoconstrictor response is however more than offset by the finding that (*a*) vascular closure often occurred making detection of graded responses impossible and (*b*) control situation was rarely regained

When inflow pressure was set at gradually higher levels vascular closure could be avoided The response however was then attenuated

In Fig 3 is presented 36 vasoconstrictor responses obtained in a group consisting of 17 lung preparations perfused at constant flow Although the stimulus is standardized the vasoconstrictor effect varied from one preparation to another This degree of inter-individual variation is well known both from studies on intact animals and man and from studies on isolated lungs (Hauge 1968*c*) The main point of the figure is however the effect of elevation in baseline pulmonary arterial pressure on the response to hypoxia There is a clear although not very marked tendency for the response to decline as the pressure is increased Fig 4 shows the same relationship obtained in a group consisting of 13 lung preparations perfused at constant [pulmo]nary arterial pressure In all 48 responses [were] evoked in this group The choice of ... different from that in the previous figure points directly to one of the main characteristics of constant pressure perfusion namely a tendency for a zero flow situation to develop at baseline pressures upwards to 3 kPa whenever a vasoconstrictor stimulus is introduced thus giving an ... large increase in PVR In comparison the ... increase in PVR in response to hypoxia at constant flow perfusion was only 190% obtained in the same range of baseline arterial pressures (... kPa) At higher pulmonary arterial pressures the constant pressure technique of perfusion [was] also more pressure sensitive This is demonstrated in Fig 5 which is a composite diagram of the responses presented so far obtained under [both] modes of perfusion In order to avoid the ... values of ΔPVR during constant pressure perfusion the abscissa starts at a pulmonary arterial pressure of 3 kPa

If we look at the pressure interspace at 3-4 ... the median increase in PVR during constant [pres]sure was 1 103% (m ... 93) a



Percentual increases in pulmonary vascular resistance caused by standardized periods of ventilation in two groups of rat lungs perfused with constant pressure technique and constant volume flow ... respectively. The responses are plotted against ... (control) pulmonary arterial pressure at (and which the responses were obtained.

flow 83 % (range 43–144) which is significantly ... wer. Also at higher pressure levels, e.g. 6–7 the difference in responses between the two ... of perfusion is highly significant: the in... in PVR being 273 % (range 122–296) and 28 % ... 27–38) for constant pressure and constant perfusion respectively. The main conclusion ... drawn from Fig. 5 is however the relatively ... influence of baseline-pressure elevation on ... onses obtained during constant flow as com... with the constant pressure perfusion ...nique.

... both modes of perfusion pulmonary arterial ...sure fluctuations with amplitudes of about 0.4 ... were observed in the control situation (no ...oxia). When the vasoconstrictor response was ...sured as reduction in flow these pressure ...ations became smaller. In two experiments ...overflow system (see Methods) was closed and ...necessary flow-reductions were done by manual adjustments of the pump-setting. The purpose was to maintain unaltered pressure-pulsations during the vasoconstrictor responses. Mean inflow pressure in these two preparations was 3.20 and 3.00 kPa, respectively. In the first preparation flow had to be reduced from 21 to 0.. ml/min during hypoxia in the other from 18 to 0.5 ml/min and from 15 to 0.2 ml/min during two periods of hypoxia. Pulse pressure amplitudes were never less than 0.4 kPa. We found no systematic differences when we compared the vasoconstrictor responses obtained with and without closed overflow system. Neither with constant nor with falling pulse-pressure amplitude was control PVR fully regained after a period of hypoxia, as long as vasoconstriction was measured as reduction in flow.

What is the effect on the response to hypoxia of elevations in venous pressure? We have attempted to answer this question by two additional experimental procedures. Firstly by left atrial pressure elevations in two of the preparations perfused at constant inflow pressure. Secondly by comparing the responses obtained during series-perfusion of two lung preparations, one anterogradely and one retrogradely, both at constant flow. A typical example of the first type of procedure is shown in Fig. 6. Pulmonary arterial pressure was maintained at 2.8 kPa, hence a fall in driving pressure and flow was induced when left atrial pressure was increased. The higher vascular transmural pressure which was established did not, however, prevent a zero flow situation when the hypoxic stimulus was introduced. The same result was obtained when the pulmonary arterial and left atrial pressures were increased to ..86 and 2.26 kPa, respectively. The other preparation tested in this manner was perfused at ... pulmonary arterial pressure of 3.19 kPa. In this preparation, flow reductions were 94 % both



Fig. 6. Flow reductions caused by two periods of ventilation hypoxia (between arrows) in a pair of rat lungs perfused at constant pulmonary arterial pressure. The horizontal line indicates a period of left atrial pressure (P_{LA}) elevation.



Fig. 3. Percentual increases in pulmonary vascular resistance caused by standardized periods of ventilation hypoxia in a group of rat lungs perfused with constant flow. Each point represents one response. The columns and bars indicate the median increase in vascular resistance and range of responses respectively, for each 1 kPa rise in baseline (control) pulmonary arterial pressure.

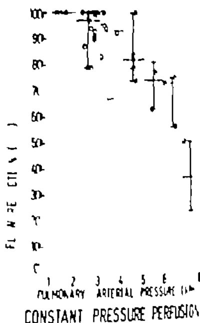


Fig. 4. Percentual pulmonary flow reductions caused by standardized periods of ventilation hypoxia in a group of rat lungs perfused with constant inflow pressure at various levels of pulmonary arterial pressure. Each point represents one response. The columns and bars indicate the median flow reduction and range of responses respectively, for each 1 kPa rise in baseline pulmonary arterial pressure.

as if a part of the vascular bed remained permanently closed.

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If we look at the pressure interspace at 3–4 kPa the median increase in PVR during constant pressure was 1103% (range ... 883) and during

higher vascular smooth muscle tension for a constrictor stimulus. This last factor may at least in part, explain why vascular closure occurs so easily in isolated rat lungs if not countered by an increase in vascular transmural pressure.

It has been debated whether critical closure of vessels, as defined by Burton (1965), occurs at all in the lungs. It has been pointed out that small muscular pulmonary arteries as defined by Harris & Heath (1977), contain relatively little smooth muscle and both an internal and external elastic lamina. Elastic tissue enough to remove the instability but not too great in amount, can still leave a great sensitivity for contractive stimuli, and allow graded responses. In some individuals and species however, acute hypoxia might induce a very high vascular smooth muscle tension so that active tension is able to win over the pressure. Regardless of the stabilizing effect of elastic tissue the radius will continue to decrease and according to the Laplace relationship less tension will then be necessary until complete closure occurs.

Humans who respond with particular vigorous pulmonary vasoconstriction when exposed to hypoxia are prone to develop high altitude pulmonary edema. There is evidence which strongly suggests that the mechanism is similar to that of pulmonary edema which accompanies severe pulmonary embolism (Hultgren & Grover 1968). In both situations occlusion of a major portion of the pulmonary vascular bed results in pulmonary hypertension. Areas of the pulmonary vascular bed that are not occluded may be subjected to abnormally high pressure and flow and unevenly distributed areas of pulmonary edema may be found. Uneven mottling of the lung fields is the usual radiographical pattern of early high altitude pulmonary edema (Penaloza & Sime 1969). Pulmonary capillary pressure as measured with wedged catheter on the arterial side is normal in these cases. These considerations do not prove that vascular closure do occur at high altitude. An alternative explanation may be that the response to hypoxia varies markedly although without actual vascular closure, from one lung region to another leading to regional variations in capillary hydrostatic pressure. Regional infinite high relative viscosity of blood in areas with low flow velocity due to vasoconstriction, is another possibility which has to be taken into account (Fischer et al. 1979). A full clarification of this question can only be made if the dimensions of the responding vessels are measured directly.

The effects of active tension on the stress-strain properties of arteriolar smooth muscle have been studied directly in vivo in the frog mesentery (Gore 1972). The arteriole was stretched by various levels of distending pressure and vessel diameter was measured with the smooth muscle relaxed. The vessel was then stimulated to contract maximally (with noradrenaline) at various levels of initial stretch and the reduction in diameter noted. The response of the vessel was markedly dependent on initial length. Thus as the distending pressure increased from low to intermediate levels the ability of the arteriole to constrict actively increased markedly but then at higher initial stress (or higher transmural pressures) the ability to constrict gradually decreased again. This relationship is similar to that found in many other types of muscle.

In the present work we found only the right hand part of the above relationship: any rise in initial transmural pressure caused reduction in the response. Thus, the maximal response appeared to occur at a lower than normal pulmonary arterial pressure in the isolated rat lung. Since the response did not seem to be much reduced at the lowest levels of stress this will enhance the likelihood of critical closure due to active tension. The situation may be different in other species and possibly also in rat lungs in situ. The in vivo autoperfused lung lobe preparations in cat (Hauge & Staub 1969, Barer et al. 1970) did however often show a zero flow condition when ventilated with N_2. An interesting observation mentioned briefly in the work of Benumof & Wahrenbrock (1975) is that in one dog a slight *increase* in the response to hypoxia was found as the left atrial pressure was elevated from 3 to 13 mmHg (0.40 to 1.73 kPa).

Individuals both human and animal with particular brisk responses to hypoxia may show less attenuation of the active constriction as the vascular diameter is reduced comparable with the response pattern found in isolated rat lungs, and experience vascular closure in some part of the lungs. The mechanism of high altitude edema as outlined above, seems to be present only in a few individuals when exposed to hypoxia. It is a rare complication, and it correlates well with the responsiveness of the pulmonary vascular bed to hypoxia.

Finally it could be argued that the observations reported in this article are due to differences in flow

when left atrial pressure was 0 and 0 93 kPa. Flow reduction was 89% when left atrial pressure was increased to 1 60 kPa.

In 14 series-perfused preparations (7 retrogradely and 7 anterogradely) baseline PVR and vasoconstrictor responses were measured in terms of inflow pressure Mean baseline inflow pressure in the "retro and antero groups were 1 46 kPa (range 1 20–2 00 kPa) and 1.33 kPa (range 0 93–2 13 kPa) respectively The median increases in PVR due to hypoxia were 100% (range 54–146) and 112% (range 33–257%) respectively Thus in spite of the large differences in left atrial and venous pressure in the retrogradely and anterogradely per fused lungs no significant differences were found as regards baseline PVR ($P=0$ 77) and the vasoconstrictor response ($P=0$ 94) In one single prepa ration perfused retrogradely at constant flow a pressor response as high as 3 86 kPa was obtained

DISCUSSION

There are two types of information to be gained from this study One is of a practical methodological kind and can be utilized when investigations of vasoactive substances and/or inhibitors are planned The other type of information is of interest in its own right since it increases our knowledge about the behaviour of the pulmonary vasculature

Reasonable demands to a test system are that the response to a given stimulus is reproducible and that a dose/response relationship can be established Isolated vascular smooth muscle strips usu ally fulfill these demands but data obtained with this method are virtually impossible to interpret in terms of vascular resistance and flow deviations Furthermore only relatively large vessels can be studied with this method Isolated perfused organs in casu lungs have several advantages Most work ers have used a constant flow perfusion technique with inflow pressure as the dependent variable As electromagnetic flowmeters have become generally available constant inflow pressure perfusion technique with flow as the dependent variable has gained in importance

We have compared the practical usefulness of the two methods by using the vasoconstrictor response elicited by acute alveolar hypoxia as a model We found as expected and in agreement with Benumof & Wahrenbrock (1975) that with rising baseline (control) vascular transmural pressure the vasoconstrictor response was reduced This was case for both methods. Since during constant perfusion the response itself is measured as pulmonary arterial pressure one might have pected that this would severely limit the usef. of this method for quantitative studies. When pared with the alternative technique it turned however that the constant flow method was superior Not only was it less sensitive to var in inflow pressure but it also had another advantage When the vasoconstrictor stimulus removed the control situation was rapidly and pletely regained making possible the application a large number of constrictor stimuli under the hemodynamic condition It has been shown that this perfusion technique gives repro dose/response relationships (Bjertnæs 19 baseline pulmonary arterial pressure with normal range

In contrast whenever the hypoxic stimulus applied under condition of constant pressure sion a permanent increase in vascular resis was seen In order to regain flow pulmonary al pressure had to be increased At normal slightly higher pulmonary arterial pressure the vasoconstriction induced with this par stimulus rapidly led to vascular closure the ing graded responses impossible to study At arterial pressures total vascular closure was vented although a permanent rise in baseline cular resistance usually occurred suggesting sure of some part of the vascular bed Expre change in vascular resistance constant pressure fusion at all levels of baseline pressure gave responses to standardized periods of vent. hypoxia. In order to demonstrate the prese absence of hypoxic vasoconstriction this may fore be the method of choice For quant studies however the constant flow per technique has several advantages It is a more robust method allowing repeated applic of the vasoconstrictor stimulus

Benumof & Wahrenbrock (1975) found that the response was usually abolished whe atrial pressure was 25 mmHg (3 33 kPa) or p nary arterial pressure about 30 mmHg (4 0 k the present work the response was present monary arterial pressures upwards to 8 kPa factors can contribute to explain this differe the vascular dimensions are different in do rats and (2) the rat is tter responder de

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ultaneous determinations of metabolic and hormonal ponses heart rate temperature and oxygen uptake nning rats

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SONNE, B. & GALBO H.. Simultaneous determinations of metabolic and hormonal responses, heart rate temperature and o ygen uptake in running rats. Acta Physiol Scand 1980 109 201–209 Received 25 Oct. 1979 ISSN 0001-6772. Institute of Medical Physiology B University of Copenhagen, Denmark.

W have developed tiny metabolism chamber including treadmill, for the study of exercising rats. The effects of work time speed and inclination of running on V_{O_2} heart rate (HR) respiratory exchange ratio (R) and rectal temperature (T_R) were determined. During prolonged running $\dot{V}_{O_2}$ HR, R and catecholamine concentrations in arterial plasma drawn through chronically implanted catheters displayed an initial overshooting which did not diminish during the four times the rats within 10 days participated in experiments. When graded exercise was performed after pre-test run of low intensity V_{O_2} HR, R and T increased with increasing running speed but did not vary with inclination During 45 min of running, the plasma concentration of glucagon increased and that of insulin remained unchanged in spite of marked increase in blood glucose. Conclusions: In exercise studies in rats it is necessary to introduce 20 min pre-test period, during which unspecific stress responses disappear This pre-test period cannot be replaced by "habituation runs on 3 preceding days. Furthermore, using rats, running speed rather than inclination has to be changed in order to establish physiologically significant differences in work intensity The chamber makes it possible to carry out sophisticated studies of adaptations to exercise of wide range of intensities in untrained rats and to relate the responses to V_{O_2}

Key words. Exercise test, adrenaline, noradrenaline insulin glucagon blood glucose homeostasis

at is extensively used as an experimental ani- studies of acute and chronic adaptations to ise. Nevertheless, there is a shortage of valid nations about the relationship between work sity and basic physiological parameters ob- d at steady state in exercising rats. This is because it is difficult to evaluate work inten- by measurements of actual and maximal O_2 uptake and partly because the small size of animal impedes sampling of blood tissues and neural signals. Studies of metabolic and hormo- responses to exercise in rats have generally been d upon sampling of blood and tissues after ise has stopped from handled anesthetized als.

The aim of the present study was to develop an experimental model (apparatus and procedure) for steady state investigations of untrained rats during controlled exercise. The effects of work time speed and inclination of treadmill running on oxygen up-

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Abbreviations used. V_{O_2} = oxygen consumption V_{CO_2} = carbon dioxide excretion, HR = heart rate T = rectal temperature, R = respiratory exchange ratio, V = flow rate of outlet air $F_{I_{O_2}}$ $F_{I_{CO_2}}$ $F_{I_{N_2}}$ = decimal fractions of O_2 CO_2 and N_2, respectively in dry inlet air $F_{O_{O_2}}$ $F_{O_{CO_2}}$ $F_{O_{N_2}}$ = decimal fractions of O_2, CO_2 and N_2, respectively in dry outlet air STPD = standard temperature and pressure dry ATPD = ambient temperature and pressure, dry NA = plasma concentration of noradrenaline, A plasma concentration of adrenaline, b.w = body weight.

pulsatility rather than the method of perfusion In the constant pressure series inflow pressure pulsations tended to become smaller during vasoconstriction whereas in the constant flow series they tended to increase The two experiments with closed overflow system and thereby unaltered pulse pressure suggest however that pressure pulsations are not a decisive factor Results from in vivo autoperfused lung lobe preparations in cat (Barer et al 1970) support this conclusion In spite of cardiogenic pulsations a zero flow condition often occurred during ventilation hypoxia

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Tables including all the relevant hemodynamic data are available from the authors on request.

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HULTGREN H N & GROVER R F 196[illegible] tory adaptation to high altitude Ann Rev [illegible] 119 152

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$\dot{V}_{O_2}(STPD)\left(\frac{F_{E_{N_2}}}{F_{I_{N_2}}} F_{I_{O_2}} - F_{E_{O_2}}\right)$

$\dot{V}_{CO_2}(STPD)\left(F_{E_{CO_2}} - \frac{F_{E_{N_2}}}{F_{I_{N_2}}} F_{I_{CO_2}}\right)$

$F_{E_{N_2}}$ was determined as $1 - F_{E_{O_2}} - F_{E_{CO_2}}$ calculated as

h

...rate was determined by counting the number of ...waves in 6

preparation

Wistar rats had one half to two thirds (about 2 ml) tails cut off by electrocoagulation in ether anesthesia in order to avoid trauma of the tail during treadmill ... No infections or growth retardation were ob... Furthermore, the squamation at the base of the ... gently scraped off in order to establish better ... between skin and tail electrodes.

... the rats had catheter (PP 50 Polystan) inserted in common carotid artery in Immobilon et. (Pharm... (0.01 ml per 100 g b.w.) anaesthesia. The following ... ure was found to be optimal resulting in 100% of ...rs functioning more than 10 days: before insertion ...heter was tapered by pulling it over flame so that ... was very thin and flexible and an enlargement was 3.0 cm from the tip using ring of electrically heated chrome wire. 3.0 cm of the heparine-saline filled ... U heparine per ml saline (0.9%)) catheter was in... in the artery and secured with ligature at each side ... enlargement. The tip was then freely floating in ... The catheter was led subcutaneously around the ... ...rting the skin between the ears. After the opera... ...ormon vet. (Pharmacia) (0.01 ml per 100 g b.w.) ...iven as Immobilon-antidote. The catheter was ...d with heparine-saline once 5 days later. 10 days ...eratively ... hen the rats were used for expts. they ...ined 17 (4–32) g (mean and range) in weight. The ...ere housed in individual cages.

...rats were accustomed to run on 3 consecutive days ... each day at speeds up to 19 m min ... In the ...ning an air jet was activated to guide them. Not all ...ere eager to run but no rats were discarded.

...mental procedure

...3 day of the actual expts. the rats were lightly ether ...hetized and for ECG-recordings clip with small ...m jack was attached to each side of thorax after ...ng the fur and applicating electrode cream. One of ...al electrodes served as ground lead. The rats with ...nically implanted catheter had saline filled exten... ...connected to the catheter by means of 2 cm of 23 ... cannula.

...h an hour after the ether anesthesia those rats, which ...ipated in exercise expts. were gently handled while ...ial electrodes were attached, the lubricated rectal ...inserted 4 cm beyond the anal sphincter and taped to the tail, and the ECG-electrodes connected. The rats were then placed on the treadmill with the belt moving at speed of 10 m min⁻¹ and an inclination of 0% in this way avoiding that the rats turned around and got themselves entangled in the wires.

8 rats (weight 209 (197–238) g in the first expt. weight gain 15 (−7–+51) g during the experimental period (10 (6–15) days)) ran four times at an inclination of 0 5 10 and 15% respectively. The individual rat always ran at the same time of the day. The order of inclinations was systematically selected to minimize influence of habituation and training. Each exercise test was initiated by 31 min at a speed of 10 m min and an inclination of 0%. Air flow $F_{E_{O_2}}$ $F_{E_{CO_2}}$ HR, T box temperature and the temperature of the outlet air were determined in the 7th 13th, 19th, 25th and 31st min of exercise. Then the inclination was set, and the speed was increased stepwise (within 0.5 min) with 5 m min every 11 min. Recordings were made in the 11th min at each work intensity. At that time $F_{E_{O_2}}$ and $F_{E_{CO_2}}$ were constant. All rats were able to perform at a speed of 20 m $\times$ min⁻¹ at all inclinations and 7 5 7 and 4 rats of 25 m min⁻¹ at an inclination of 0 5 10 and 15% respectively. Only one rat was able to run at a speed of 40 m min.

The day after the last run each of the 8 rats was subjected to a swim in an air-tight plexiglas cylinder (19 cm d. 38 cm high) filled with water (33 °C and equilibrated with atmospheric air) to depth of 36 cm. $\dot{V}_{O_2}$ was determined using the same principle as described for the treadmill with an air flow of approximately 1000 ml (ATPD) min⁻¹. The rats swam in 6 min periods with increasing tail weights (0–11 g) until $\dot{V}_{O_2}$ levelled off or decreased.

In the morning after 1–2 h without access to food the 18 catheterized rats (weight 243 (220–262) g) either rested for 45 min or ran at an inclination of 0% 15 min at speed of 10 m min⁻¹ followed by 30 min at speed of 14 m min. Recordings were made in the 5th, 15th, 30th and 45th min of rest and exercise respectively (in rest expts. T was only measured immediately before the start of the experimental period). In the 5th 15th and 30th min 0.7 ml of blood for catecholamine analysis was drawn from the catheter into iced test tubes containing 1.4 mg EDTA and 1.4 mg ascorbic acid discarding the saline filling the catheter. The blood removed was replaced by blood obtained in heparinized syringe by heart puncture of donor rat immediately before the expt. Thereafter the catheter was refilled with saline. In the 45th min 0.7 ml of blood was drawn for catecholamine analysis and then 2.5 ml was drawn for analysis of glucose and lactate, and for analysis of plasma insulin and glucagon (2 ml into iced test tubes containing 100 µl Trasylol (Bayer)). Adrenaline (A) and noradrenaline (NA) were determined by radioenzymatic micromethod, and glucose (hexokinase) and lactate by enzymatic spectrophotometric methods. Insulin and glucagon were determined by radioimmunoassays using rat insulin and pork glucagon as standards.

Statistical analysis of the data was performed using the Wilcoxon rank sum test for paired and the Mann-Whitney rank sum test for unpaired data. Correlation coefficients were calculated and compared according to Snedecor & Cochran (1965).

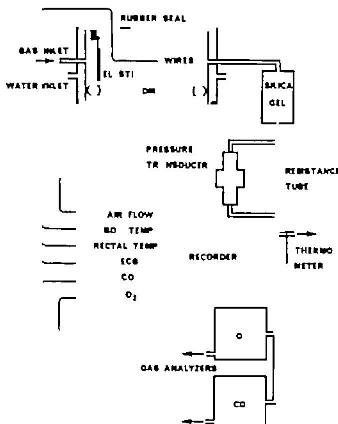


Fig 1 Schematic diagram of metabolism chamber and auxiliary equipment. EL. STIM displaceable plate and microswitch

take (V_{O_2}) heart rate (HR) respiratory exchange ratio (R) and rectal temperature (T_R) were determined in order to establish an experimental procedure for attainment of valid informations about responses to different levels of exercise in rats Since levelling off of oxygen uptake was not found we evaluated if the highest oxygen uptakes measured during running were really the highest obtainable during exercise by comparing them to oxygen uptakes measured during forced swimming Finally chronically implanted arterial catheters were used for blood sampling from unanesthetized rats at rest and during running making it possible optimally to measure metabolic and hormonal responses to exercise

MATERIALS AND METHODS

Description of apparatus (Fig 1)

The system consists of a tiny motordriven treadmill the speed of which can be continuously varied between 0 and 80 m min A velocimeter with digital display makes it possible to adjust the speed accurately The motor is placed outside and the treadmill inside a [illegible] metabolism chamber (inside dimension [illegible] 13 cm) the inclination of which can be continuously [illegible] from 0 to 15% allowing studies during uphill [illegible] possible to establish a flow of water between the [illegible] (space 1900 cm³) thus regulating the temperature [illegible] box in expts where extreme ambient temperatures [illegible] quired A thermistor 1 cm below the lid measures temperature in the box.

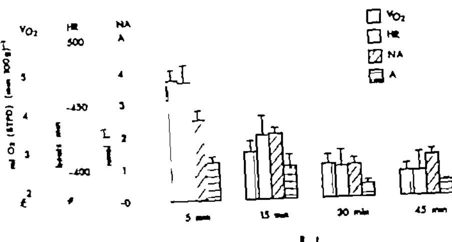
An adjustable flow of tank gas (in all the present [illegible] atmospheric air) is led into the box at the rear end [illegible] treadmill The outlet air passes through coarse [illegible] for dehydration and further through a resistance [illegible] (13 gauge 29 cm) over which the pressure [illegible] measured by a strain gauge differential pressure [illegible] ducer The system is calibrated using known [illegible] and serves as a pneumotachograph Flow rate [illegible] to approximately 1500 ml (ATPD) min [illegible] expts and in the initial 6 min of rest expts [illegible] flow rate of approximately 1000 ml (ATPD) [illegible] used from the 7th min onward in rest expts [illegible] resulting in box pressures 0 8 and 0.5 kPa above [illegible] metric pressure respectively and in a CO_2 concentration in the box air below 1% Immediately after the [illegible] tube 600 ml (ATPD) min [illegible] is withdrawn from [illegible] gas analysis whereas the excess air passes [illegible] meter An oxygen analyzer (Applied Electrochemistry S-3A oxygen analyzer with a N 22M oxygen sensor [illegible] a R 1 flow control) and a carbon dioxide analyzer [illegible] man LB-2) are connected in parallel The air flow [illegible] the analyzers are 100 and 500 ml (ATPD) [illegible] ly yielding the optimal accuracy for both [illegible] The gas-analyzers are calibrated before each expt [illegible] tank gases with approximately 21 and 17% O [illegible] 4% CO All tank gases are analyzed by the [illegible] technique The electronic analyzers are stable for [illegible]

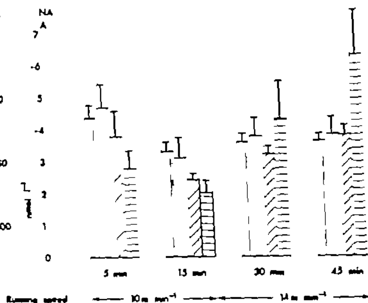
The rats are encouraged to run by mild electrical [illegible] lation A plexiglas ring (1 0 cm i.d 1 4 cm o d) [illegible] small cylinder head machine schrews encloses the [illegible] the tail If the rat stops running it bumps into a [illegible] able plate at the rear end of the treadmill A microswitch [illegible] turned off and the rat receives a constant [illegible] through the tail electrodes as long as the plate is [illegible] The voltage can be varied from 0 to 40 V

The wires for the tail electrodes enter the box [illegible] the lid at the rear end of the treadmill together [illegible] wires for the box thermistor for a rectal thermistor [illegible] for two small banana plugs used for ECG-recording [illegible] disposable pierceable rubber seal makes it possible [illegible] catheters air tight through the lid The outputs [illegible] pneumotachograph the gas-analyzers and the [illegible] rectal thermistor are recorded on a six-channel [illegible] (Chessel 3?0) Using locally constructed amplifiers [illegible] recorder is calibrated to give deflections of 1 cm [illegible] ml (ATPD) min 1 cm per 0 2% of O_2 and CO_2 [illegible] per 0.2°C (rectal thermistor) and 1 cm per [illegible] thermistor) The ECG-signal is recorded on an Ultralette 5651 at a paper speed of 75 cm min

Calculations

V_{O_2} and V_{CO_2} were calc





V_{O_2}, HR, noradrenaline (NA) and adrenaline (A) during 45 min of rest or running at an inclination of 0%. Values m ± S E for 9 rats

...ral temperature

...mbient temperature in the box was at the start ... expts, 23.0 (19.8–28.0) °C and it increased 1.8 ... 3 5) °C during running and 0.6 (0.2 1 4) °C in ... expts.

...d 31 min of exercise (Fig 2)

... decreased ($P<0.05$) from the 7th to the 13th ... thereafter it remained unchanged HR and R decreased from the 7th to the 19th min of exercise ($P<0.05$) whereafter they remained unchanged. T did not change significantly during the 31st min of running.

Neither V_{O_2} HR, R nor T changed significantly from the first to the fourth time the rats participated in exercise expts. Comparison of the two regression lines for HR (y) versus V_{O_2} (x) calculated at the 7th (y=365+19x ... =31) and 31st min (y=333+20x

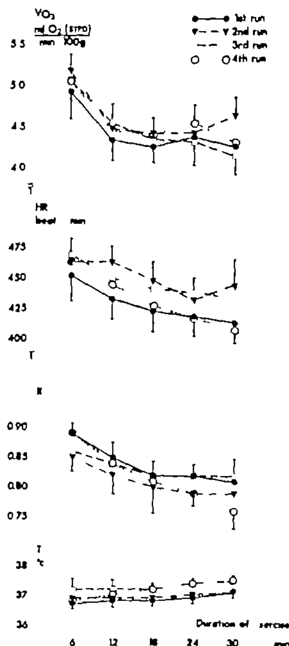


Fig 2 V_{O_2} HR R and T during four runs on separate days at a speed of 10 m min and an inclination of 0%. Values are mean ± S E for 8 rats.

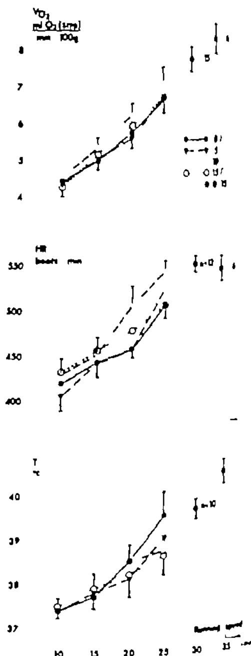


Fig 3 V_{O_2} HR and T as a function of running speed inclinations. Values are mean ± S E for 8 rats

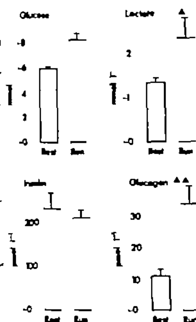
RESULTS

Kinetics

A series of expts was performed to evaluate the mixing of the air in the metabolism chamber 100% N_2 was introduced in the chamber at different flow rates and F_{O_2} was registered continuously When the treadmill belt was not moving a biexponential wash out curve occurred (k_1=0 7? and k_2=0 28 min $^{-1}$ at a flow rate of 1475 ml (ATPD) min $^{-1}$) But when the belt was moving at a speed of 10 m min $^{-1}$ the wash out curve was monoexponential with a $T_{\frac{1}{2}}$ which at the flow r used in the expts agreed exactly with the $T_{\frac{1}{2}}$ culated as

$$\ln 2 \cdot \frac{\text{volume of air in chamber (1900 cm}^3)}{\text{flow rate}}$$

indicating complete mixing of the air in chamber When the rat volume is taken into count $T_{\frac{1}{2}}$ can be calculated to 1... min using above formula at a flow rate of 1400 (ATPD) min



Blood glucose and lactate concentrations and insulin and glucagon concentrations in the 45th ... rest or running (15 min at ... running speed of 10 ... followed by 30 min at 14 m min⁻¹ at an inclina- ...0%). Values are mean ± S.E. for 9 rats. Δ denotes ..., ΔΔ denotes $P<0.01$

...olamine concentrations (Fig. 4). Accordingly ...tial peculiar finding of relatively high V_{O_2} at ...w speeds in the beginning of the graded exer... ...est probably was an effect of catecholamines ...ed as an excitement reaction to handling and a ...nvironment. Increases in the plasma concen-...ns of catecholamines upon handling and re-...t have previously been found in the rat (Pop-... al. 1977 Kvetnansky et al. 1978). The initial ...shooting in V_{O_2} and HR did not diminish ...g the four times the rats within a period of 10 ... participated in exercise expts (Fig. 2). A simi-...r that overshooting was found in rats studied ...st (Fig. 4) V_{O_2} initially being equal to V_{O_2} ...ng steady state running at about 13 m min ... consequence of the above findings we found it ...ssary to begin each exercise test with a 31 min ...tation period. A habituation period of slow ...ing was chosen rather than a period of rest ...h the rats at rest would get themselves en-...led in the wires. Furthermore the switch on of ...e treadmill probably would elicit excitement reactions. After the introduction of a pre-test period the responses to exercise were as expected. V_{O_2}, HR, R and T increased with increasing speed of running (Fig. 3). In the light of our findings it seems likely that many previous measurements carried out in exercised rats reflect excitement reactions as well as responses to exercise, e.g. this was probably the case in a recent study in which cardiovascular parameters were measured after 5 min of running in rats which had two arterial catheters implanted 3 h in advance and which had no prior treadmill ex perience (Flaim et al. 1979).

At the end of each graded exercise test the rats ran at a speed which they were only able to cope with for a period of time which as a consequence of the response time of the whole system, was too short to allow measurement of the actual $\dot{V}_{O_2}$. The response time might be shortened by an increase in flow rate but then the accuracy of the readings of the gas analyses would decrease. However during the few minutes at the highest speed F_{O_2} and F_{CO_2} changed to values lower and higher respectively than those obtained at lower speeds. This indicates that the highest measured V_{O_2} was not maximal a supposition supported by the fact that V_{O_2} and HR did not level off at the highest speeds (Fig. 3). However the highest V_{O_2} which we were able to measure during running was probably nearly maximal. This is so since it was higher than the highest V_{O_2} measured during forced swimming, the latter agreeing with previously reported values (McArdle 1967). Furthermore it was identical to maximal V_{O_2} defined by a levelling off criterion, in untrained, running (Wilson et al. 1978) or cold exposed (Rosenmann & Morrison 1974) rats.

T was higher during running than at rest and increased with running speed in these expts. (Fig. 3) in which the ambient temperature was within the zone of thermoneutrality as defined for rats (Poole & Stephenson 1977). It could be argued that the lack of a part of the tail might render heat loss difficult and thus augment the T response to exercise. However it can be calculated that the removed part of the tail at the most amounted to about 6% of the total non-evaporative heat dissipation capacity in the rat (Rand et al. 1965). Furthermore when core temperature during exercise exceeds 39 °C evaporation of saliva spread over chest and forelegs probably aids in heat dissipation, and the tail accordingly accounts for a smaller fraction of the total heat loss than during non-evaporative

Table 1 *Correlation coefficients between $\dot{V}_{O_2}$, HR, T_R and running speed at inclinations of 0, 5, 10 and 15%*

Inclination	0%	5%	10%	15%
$\dot{V}_{O_2}$ and speed	0 80 (39)	0 88 (34)	0 80 (35)	0 74 (32)
HR and speed	0 68 (32)	0 86 (23)	0 76 (25)	0 83 (25)
HR and $\dot{V}_{O_2}$	0 76 (3?)	0 85 (23)	0 78 (25)	0 79 (25)
T_R and speed	0 74 (31)	0 73 (25)	0 71 (22)	0 69 (29)
T_R and $\dot{V}_{O_2}$	0 82 (31)	0 82 (25)	0 86 (22)	0 66 (29)

All correlation coefficients are statistically significant ($P<0\ 001$) Correlation coefficients obtained at different inclinations are not significantly different from each other Number of observations is shown in parentheses

$n=31$) revealed that the regression lines may be regarded as parallel but with different intercepts ($P<0\ 001$) indicating that in the 7th compared to the 31st min HR is relatively more increased than $\dot{V}_{O_2}$

Effects of running speed

$\dot{V}_{O_2}$ and HR increased with increasing running speed ($P<0\ 05$) at all inclinations (Fig. 3) Positive correlations between $\dot{V}_{O_2}$ and speed HR and speed and between HR and $\dot{V}_{O_2}$ ($y=?98+30x$ $n=135$) were found at each inclination (Table 1) The highest measured $\dot{V}_{O_2}$ and HR were 8 6±0 3 ml O_2 (STPD) (min 100 g) (mean±S E $n=8$) and 556±16 beats min ($n=8$) respectively

At the end of the initial 31 min period the R value was 0 81±0 02 ($n=23$) At a running speed of 25 m min R increased to 0 84±0 01 ($n=23$) ($P<0\ 0?$) A positive correlation between R and running speed was found ($r=0\ 36$ $P<0\ 001$) The highest measured R was 0 9?±0 02 ($n=8$)

T_R increased with increasing running peed at all inclinations (Fig 3) Compared to the values obtained at a speed of 10 m min the increase was significant at 20 m min ($P<0\ 05$) Positive correlations between T_R and speed and between T_R and $\dot{V}_{O_2}$ were found at each inclination (Table 1) The highest measured T_R was 40 0±0 4 °C ($n=8$)

Effects of inclination

No significant differences in $\dot{V}_{O_2}$ HR R and T were found when comparing measurements at different inclinations and identical speeds obtained in expts carried out on different days (Fig 3) Furthermore when comparing values obtained a 31st min of pre-test running at a speed of [illegible] min and an inclination of 0% to values obt 10 min later at the same running speed and inclination of 5 10 or 15% no influence of the inclination on these parameters could be demonstrated

$\dot{V}_{O_2}$ during swimming

The highest measured $\dot{V}_{O_2}$ during running was always higher (?6 (3–59) %) than the highest measured $\dot{V}_{O_2}$ during swimming (6.9±0 2 ml (STPD) (min 100 g)$^{-1}$)

Rest (Fig 4)

$\dot{V}_{O_2}$ HR and the plasma concentrations of NA A decreased during the initial 30 min of ($P<0\ 01$) and then remained unchanged $\dot{V}_{O_2}$ correlated with NA ($r=0\ 66$ $P<0\ 001$) in the [illegible] min of rest T_R was 36 7 (35 9–37 9) °C.

Metabolic and hormonal responses to exercise

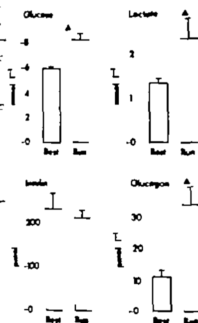
At comparable times A and NA were higher during prolonged running than at rest ($P<0\ 05$) (Fig 4) and NA ($P<0\ 05$) decreased during the [illegible] min of prolonged running and then [illegible] ($P<0\ 02$) with $\dot{V}_{O_2}$ (Fig 4) During the first [illegible] of running A ($r=0.55$ $P<0\ 05$) and NA (r [illegible] $P<0\ 10$) correlated with $\dot{V}_{O_2}$

Compared to the values obtained in the [illegible] of rest blood glucose and lactate concentrations [illegible] the plasma glucagon concentration increased during running whereas plasma insulin concentration remained unchanged (Fig 5)

DISCUSSION

In the present paper a metabolism chamber for study of running rats is described A procedure [illegible] attainment of valid information about responses to exercise in the rat was developed and used to characterize some metabolic and hormonal reactions

In our preliminary expts we found to our surprise that $\dot{V}$ decreased with increasing speed of running during an uninterrupted graded exercise test Further investigations showed that $\dot{V}_{O_2}$ as well as HR HR/$\dot{V}_{O_2}$ and R decreased during the first min of exercise at a constant work load These changes were accomp[anied] by decreasing [illegible]



Blood glucose and lactate concentrations and insulin and glucagon concentrations in the 45th ... rest or running (15 min at ... running speed of 10 ... followed by 30 min at 14 m min ... at an inclination ... %). Values are mean ± S E for 9 rats Δ denotes ... ΔΔ denotes $P<0.01$

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V_{O_2} HR and the plasma concentrations of NA and A decreased during the initial 30 min of rest ($P<0\ 01$) and then remained unchanged V_{O_2} correlated with NA ($r=0\ 66$ $P<0\ 001$) in the [illegible] min of rest T_R was 36 7 (35 9–37 9) °C

Metabolic and hormonal responses to running

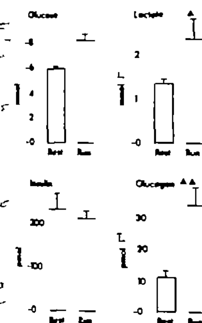
At comparable times A and NA were higher during prolonged running than at rest ($P<0.05$) (Fig 4) and NA ($P<0\ 05$) decreased during the [illegible] min of prolonged running and then [illegible] ($P<0\ 0?$) with V_{O_2} (Fig 4) During the first [illegible] of running A ($r=0\ 55$ $P<0\ 05$) and NA ($r=$ [illegible] $P<0\ 10$) correlated with V_{O_2}

Compared to the values obtained in the [illegible] of rest blood glucose and lactate concentrations [illegible] the plasma glucagon concentration increased during running whereas plasma insulin concentration remained unchanged (Fig 5)

DISCUSSION

In the present paper a metabolism chamber for study of running rats is described A procedure for attainment of valid information about responses to exercise in the rat was developed and used to characterize some metabolic and hormonal [illegible] tions

In our preliminary expts we found to our surprise that V_{O_2} decreased with increasing speed of running during an uninterrupted graded exercise test Further investigations showed that V_{O_2} as well as HR HR/V_{O_2} and R decreased during the first min of exercise at a constant work load These changes were accomp[anied] by decreasing [illegible]



Blood glucose and lactate concentrations and insulin and glucagon concentrations in the 45th test of running (15 min at running speed of 10 ... followed by 30 min at 14 m min ... at an inclina- ...%) Values are mean ± S E for 9 rats. Δ denotes ... ΔΔ denotes $P<0.01$

...olamine concentrations (Fig. 4) Accordingly ...ial peculiar finding of a relatively high V_{O_2} at ... speeds in the beginning of the graded exer... ...st probably was an effect of catecholamines ...ed as an excitement reaction to handling and a ...nvironment Increases in the plasma concen... ...ns of catecholamines upon handling and re... ...t have previously been found in the rat (Pop... ...al 1977 Kvetnansky et al 1978) The initial ...shooting in V_{O_2} and HR did not diminish ... the four times the rats within a period of 10 ... participated in exercise expts. (Fig. 2) A simi... ...itial overshooting" was found in rats studied ... (Fig 4) V_{O_2} initially being equal to V_{O_2} ... steady state running at about 13 m min ... consequence of the above findings we found it ...essary to begin each exercise test with 31 min ...uation period. A habituation period of slow ...ing was chosen rather than a period of rest ... the rats at rest would get themselves en... ...led in the wires. Furthermore the switch on of ... treadmill probably would elicit excitement

reactions. After the introduction of a pre-test period the responses to exercise were as expected V HR R and T increased with increasing speed of running (Fig. 3) In the light of our findings it seems likely that many previous measurements carried out in exercised rats reflect excitement reactions as well as responses to exercise e g. this was probably the case in a recent study in which cardiovascular parameters were measured after 5 min of running in rats which had two arterial catheters implanted 3 h in advance and which had no prior treadmill ex perience (Flaim et al. 1979)

At the end of each graded exercise test the rats ran at a speed which they were only able to cope with for a period of time which as a consequence of the response time of the whole system was too short to allow measurement of the actual V_{O_2} The response time might be shortened by an increase in flow rate but then the accuracy of the readings of the gas analyses would decrease. However during the few minutes at the highest speed F_{O_2} and F ... changed to values lower and higher respectively than those obtained at lower speeds. This indicates that the highest measured V_{O_2} was not maximal a supposition supported by the fact that V_{O_2} and HR did not level off at the highest speeds (Fig. 3) However the highest V_{O_2} which we were able to measure during running was probably nearly maximal This is so since it was higher than the highest V_{O_2} measured during forced swimming the latter agreeing with previously reported values (McArdle 1967) Furthermore it was identical to maximal V_{O_2} defined by a levelling off criterion in untrained, running (Wilson et al 1978) or cold exposed (Rosenmann & Morrison 1974) rats

T was higher during running than at rest and increased with running speed in these expts. (Fig 3), in which the ambient temperature was within the zone of thermoneutrality as defined for rats (Poole & Stephenson 1977). It could be argued that the lack of a part of the tail might render heat loss difficult and thus augment the T response to exercise However it can be calculated that the removed part of the tail at the most amounted to about 6% of the total non-evaporative heat dissipation capacity in the rat (Rand et al 1965) Further more when core temperature during exercise exceeds 39 °C evaporation of saliva spread over chest and forelegs probably aids in heat dissipation, and the tail accordingly accounts for a smaller fraction of the total heat loss than during non-evaporative

conditions (Gollnick & Ianuzzo 1968 Poole & Stephenson 1977) The supposition that heat dissipation was not severely impeded is supported by the fact that T_R at rest and during exercise was slightly lower than previously reported for rats at similar ambient temperature (Gollnick & Ianuzzo 1968) However this discrepancy may also to some extent be due to the fact that T_R had not always plateaued when read in the present expts At rest also differences as regards the distance from the anal sphincter at which T_R is measured (4 cm in the present vs 5.5 cm in the cited study) may explain the diverging T_R values (Lomax 1966) The relationship between site of measurement and T_R in the rat during exercise is unknown

At constant speed of running treadmill inclination did not influence V_{O_2} HR R and T_R in the present expts This supports the conclusion that running uphill involves a relatively smaller increase in energy expenditure over horizontal running for small than for large animals (Taylor et al 1972)

During prolonged exercise the plasma concentration of glucagon increased and that of insulin remained unchanged in spite of a marked increase in blood glucose concentration (Fig 5) We have previously found a similar pattern in swimming rats (Richter et al 1979) However at that time the concentrations were measured after termination of exercise and it could not be excluded that a post exercise change had occurred From studies carried out in dogs it has been claimed that during prolonged exercise the plasma glucose concentration is a precisely regulated variable which is kept constant in spite of large increases in glucose production and utilization (Vranic et al 1976) It appears from the present study that in the rat the blood glucose concentration may indeed increase during prolonged exercise indicating that at least in this species hepatic glucose production is not accurately adjusted to peripheral glucose utilization on a moment to moment basis during exercise

In conclusion the present study has shown that in order to carry out work physiological investigations in rats it is necessary to introduce a 20 min pre-test period during which unspecific stress responses to handling and a new environment disappear This pre-test period cannot be replaced by "habituation runs on three preceding days Furthermore using rats running speed rather than inclination has to be changed in order to establish physiologically significant differences in work intensity Taking these findings into account the developed metabolism chamber makes it possible to carry out sophisticated studies of metabolic, hormonal and cardiovascular adaptations to exercise intensities almost up to maximal aerobic capacity in untrained rats and to relate the responses to O₂ uptake

This investigation was supported by grants from the Carl Petersen Foundation Landsforeningen for Sukkersyge Idrættens Forskningsråd and Fru P A [illegible] Legat. Novo Research Institute kindly donated [illegible] insulin analysis John Jonson constructed the metabolism chamber Dr N J Christensen, Department of Internal Medicine and Endocrinology Herlev Hospital, Copenhagen carried out the catecholamine analyses [illegible] beth Kall and Dr E A. Richter performed skilled technical assistance

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...d flow in arteries determined transcutaneously ...n ultrasonic doppler velocitymeter as ...pared to electromagnetic measurements ...he exposed vessels

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GULDVOG L, KJÆRNES M, THORESEN M & WALLØE, L. Blood flow in arteries determined transcutaneously by an ultrasonic doppler velocitymeter as compared to electromagnetic measurements on the exposed vessels. Acta Physiol Scand 1980 109 211–216. Received 25 Oct 1979 ISSN 0001-6772. Institute for Surgical Research, Rikshospitalet, University of Oslo Institutes of Physiology and Informatics, University of Oslo and Institute of Physiology University of Oslo Norway

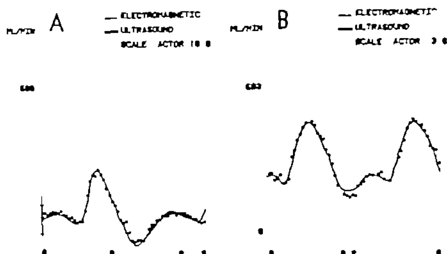
W have compared the instantaneous mean blood velocity measured transcutaneously from total cross-sections of arteries in dogs by the use of new pulsed heterodyne ultrasound doppler equipment, with instantaneous blood flow as measured simultaneously by an electromagnetic flow meter applied on the exposed vessels. The experiments were per formed on the common carotid and the femoral arteries in a variety of flow situations, always with close fit between the two curves. The results show that the output from the ultrasound equipment is proportional to flow

early 1960's Rushmer and collaborators de... an ultrasonic doppler instrument which ...measure blood velocities through the skin ...ushmer et al. 1966). Theoretically the dop...gnal from such an instrument should contain ...ation about all velocities present in the ves... all parts of the cross-sectional area of the ...are illuminated by a soundfield of constant ...ty the back-scattered signal should also con...formation about the relative amount of blood ...g at each of these velocities. Thus in their ...st papers, Rushmer and collaborators indi... the possibility of non-invasive volume flow ...rements by means of a doppler instrument.

...ny attempts have been made to improve the ...al instruments (see Woodcock 1975 for refer...). However the volume flow measurement ...has not yet been reached. This article presents ... attempt in the direction of volume flow ...urements.

...have tried to improve the existing ultrasound equipment in three different ways. (1) The ultrasound probes have been carefully designed built and then measured to ensure an approximately constant sound-field with sufficient width. (2) A new doppler instrument has been constructed (Hatteland & Eriksen 1980) with sharp filters in order to remove stationary echos and doppler frequencies caused by movements of the vessel wall without seriously affecting doppler frequencies caused by the moving blood. This instrument has a heterodyne audio output. (3) All estimates (e g. of the mean doppler frequency across the vessel lumen) are made from frequency spectra which are calculated from the heterodyne audio output in a computer system (Wille 1977) Thus biased estimators such as the zero-crossing estimator are avoided, and it is easy to make the estimators robust against noise

The aim of our project so far has therefore been to estimate the true mean doppler frequency across the vessel lumen and its variation with time To



Comparison of the electromagnetic flowrecording and the best fitted ultrasound blood velocity measurements two different vessels in one dog (A) Femoral artery 6 MHz. (B) Common carotid artery 6 MHz In both panel horizontal axis is time in s, and the vertical axis is flow in ml/min as determined by the electromagnetic flowmeter below the horizontal axis correspond to backflow The two panels were obtained from the computer by means of matic plotter The pulse frequency was little lower during the femoral recording than during the carotid ing Assuming measurement angle of 45° scale factor 10 0 correspond to internal diameter 2.48 mm, and scale 13 8 corresponds to internal diameter 2.97 mm.

Velocities towards the transducer cause frequencies above the chosen base-line and velocities away the transducer cause frequency shifts below the chosen base-line frequency UNIDOP contains 12 pole low Butterworth filters adjustable to four different sampling frequencies with break point at 40 % of the sampling frequency In addition one can choose from among three highpass Butterworth filters with break frequencies at 200 Hz, 400 Hz and 700 Hz. These filters will eliminate frequencies caused by vessel wall movements and other disturbances.

The velocitymeter is connected to an analog real-time compression spectrumanalyzer in our set-up It is helpful to see the spectrum on scope simultaneously with hearing the audiosignal from the loudspeaker One use of the real time spectrum is to adjust the amplitude threshold to get rid of most of the noise without losing too much of the informationcontaining signal. Another use of the spectrum is to discriminate between vessels depending on the characteristic shape of their spectra

To store the audiosignal for later calculations, the UNIDOP is connected to stereo casette tape recorder (Akai 4760 D) The doppler signal is recorded on one channel and the ECG and comments are recorded on the other channel. Both the tape recorder and the velocitymeter is connected on-line to NORD computer system. For practical reasons we usually store the recordings on magnetic tape and play the tape back for later calculations in the computer The computer may calculate the spectrum as frequently as every 8 ms, using fast fourier transform algorithm, and also the mean velocity from each spectrum (... 1977)

Since we want to measure the relative intensity of all velocities present in the whole cross-section of the vessel,

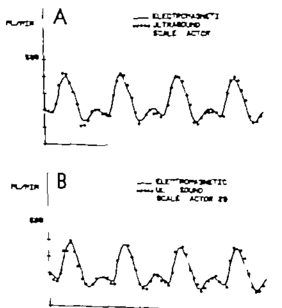


Fig 3 Ultrasound blood velocity measurements as compared to electromagnetic flow recordings. (A) Common carotid artery 6 MHz. (B) Common carotid artery 1.5 MHz. Axes and symbols as in Fig 2. A new mean doppler frequency is calculated each 20 ms. In panel A scale factor 14.3 corresponds to internal diameter 2.96 mm and in panel B scale factor 29.5 corresponds to internal diameter 3.36 mm.

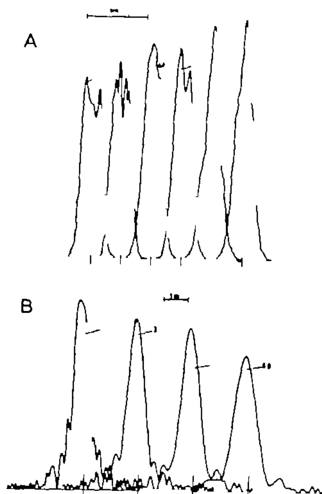


Fig 1 Sound fields emitted from the two ultrasound transducers. (A) Intensity distributions in front of the 6 MHz ultrasound transducer as measured normally to the beam axis in 6 different distances from the crystal surface in a water tank The intensity was measured by a small ceramic crystal mounted on the tip of a needle The effective diameter of the probe was 1 4 mm Horizontal axis distance across the field Vertical axis sound intensity the scale is arbitrary but is the same for all distributions The 6 distributions are displaced along the horizontal axis for clarity (B) Intensity distributions in front of the 1 5 MHz ultrasound transducer Axes etc as in A

convert this mean doppler frequency to mean blood velocity either an a priori knowledge or a measurement of the angle between the sound beam and the velocity vector is necessary The instantaneous blood flow through the vessel is equal to the mean blood velocity multiplied by the cross-sectional area of the vessel Thus knowledge of or measurement of both the angle and this area are necessary to convert the mean doppler frequency to volume flow The problems connected with measurements of the angle and of the cross-sectional area have not been approached in this study However in situations where the measurement angle and the c sectional area of the vessel can be assumed approximately constant, changes in the mean doppler frequency with time for example during cardiac cycle should be proportional to the flow In the present study we have compared mean doppler frequencies obtained transcutaneously by use of our equipment in dogs with flow as measured simultaneously by an electromagnetic flowmeter applied on the exposed vessels

METHODS

Surgical and experimental procedures

The experiments were performed on 3 seven ... golden retriever dogs (weight approximately ...) The animals were anesthetized with i v injection of ... pentobarbital (Nembutal) During the experiment ... was recorded by needle-electrodes, and blood pressure by means of an intra arterial catheter The femoral ... were exposed by short transversal section in the ... the electromagnetic flow probes were applied to ... free-dissected segment of the vessel. The ultrasound ... was aimed at the vessel 1–2 cm upstream from the electromagnetic flow probe through the shaved skin. The ultrasound probe was held by hand at an angle to the ... approximately 45° Based on knowledge of the vascular anatomy of the dog we tried to choose ... of the femoral artery without any branches between two measurement areas The common carotid ... were exposed in the same manner 5 cm beneath the ... of the jaw However since the common carotid does not have any branches the ultrasound measurements could be performed both cranially near the ... electromagnetic flow probe and closer to the ... where the vessel runs deeper

The ultrasound doppler velocitymeter

The doppler velocitymeter UNIDOP used in the investigation and part of the computer system ... data analysis have been described in detail else... (Hatteland & Eriksen 1980 Wille 1977). Velocity meas... urements can be obtained from most vessels in the ... as long as the sound beam does not hit bone or gas (lungs or parts of the intestines) To take measure... on deep vessels down to the depth of 10 cm the frequency 1.5 MHz is used which gives a good penetration. Recording from superficial vessels ... .5 cm one uses 6 MHz, which gives a better ... resolution In the present investigation 6 MHz was used on the femoral artery and on the cranial part ... common carotid artery while 1.5 MHz sound was used ... the central part of the common carotid artery Both frequencies can be operated either in a pulsed ... or continuous wave mode The pulsed mode was used in the present investigation The pulse lengths were ... lengths

UNIDOP uses heterodyne detection to discriminate between forward and backw... ow The base-band ...

2. Repeated doppler elocity measurements common carotid rtery as compared to elec -netic flow measurements

rasound probe was removed completely between easurement Observations no. 5 and 6 were ob- on closer to the heart than the first four

EM-flow (ml/min)	Scale factor	Doppler flow (ml/min)	Calculated radius (mm)
226 2	14 5	218.3	1 49
212 2	14 8	206.5	1.51
238 3	13 8	234 6	1 46
232 4	14.6	228.7	1 50
205 1	17 0	200 6	1 6..
190 2	17 9	184.5	1.66

A the cross-sectional area of the vessel If both ss-sectional area A and the measurement angle θ assumed to be constant, the mean doppler frequen- the instantaneous flow should be proportional. As approximation we assume that these conditions are d during one pulse cycle. Consequently instan- ous flow and instantaneous mean blood velocities were compared during corresponding cardiac cycles. comparison procedure has been as follows Parts of recordings where both the electromagnetic flowmeter trasound velocitymeter the arterial pressure meas- nts, and the ECG were operating properly were ed From the ultrasound recording, the mean dop- requencies were calculated each 8 ms through one or cardiac cycles as described in Wille (1977) The sponding recording from the electromagnetic meter was sampled in exactly the same points in time. computer then determined the proportionality factor ssary to obtain the best fit between the velocity re- ng and the flow recording A least mean absolut ation between the two curves was the criterion for fit. In theory the proportionality factor is function e cross-sectional area of the vessel and of the meas- ent angle Assuming an angle of 45° and assuming lar cross-section of the vessel it is possible to calcu- the internal diameter of the vessel corresponding to best fit between the two curves

RESULTS

2 shows the results obtained in two vessels in same dog. An emitting frequency of 6 MHz was d for the ultrasound measurements both on the moral artery and on the common carotid artery g. 3 shows tw similar pairs of curves. Both els represent measurements on the common otid artery The upper panel displays ultrasound measurements obtained near the flow transducer by means of 6 MHz ultrasound The lower panel displays measurements obtained deeper on the neck near the thorax by 1.5 MHz ultrasound A and B are not simultaneous recordings, but were obtained from the same dog

Fig. 4 shows the results obtained from a femoral artery before (A) and after (B) an intra arterial injection of papaverin (20 mg) In Fig. 4 A the average flow during one cardiac cycle is 45.5 ml/min and in Fig 4 B the corresponding flow is 110 0 ml/min

Figures such as those displayed in Figs 2 3 and 4 were obtained in all three dogs in a variety of flow situations Towards the end of each experiment, either papaverin or bradykinin was injected with large vasodilatation as the result. The fit between the electromagnetic flow and the ultrasound blood velocity was equally good in these flow situations.

A few longer series of recordings were obtained for statistical purposes.

In some series the ultrasound transducer was kept in position continuously for more than half a minute Table 1 shows the results from one such series. The best fit to the electromagnetic flow-recording was obtained individually for each heart beat The table illustrates the typical degree of scatter between successive cardiac cycles

In other series the ultrasound transducer was removed completely from the skin while the investigator was busy with other tasks. Afterwards the transducer was aimed in at the vessel again This procedure was repeated a number of times Table 2 shows the results from one such series

DISCUSSION

The results show that a very good fit can be obtained between our ultrasound blood velocity measurements and the electromagnetic flow measurements. However as long as the good fit is obtained partially by means of the arbitrary scale factor we are somewhat reluctant to draw too firm conclusions Clearly the results would have been more convincing if both the cross-sectional area and the measurement angle had been measured during the experiment and the subsequent fit was obtained without any curve fitting. However precise measurements of the internal cross-sectional area of a vessel under different physiological and pharmacological conditions is research project in its

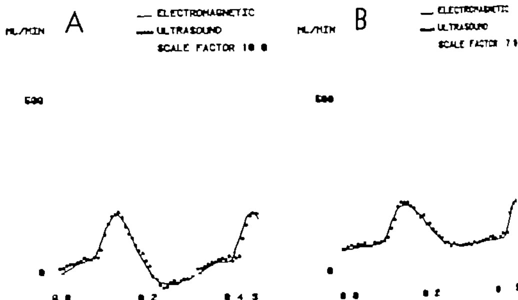


Fig 4 Ultrasound blood velocity measurements as compared to electromagnetic flowrecordings. (A) Femoral art MHz. (B) Femoral artery 6 MHz, after injection of papaverin Scale factor 10 0 corresponds to internal diame mm. Scale factor 7 9 corresponds to internal diameter 2.20 mm Blood pressure in A was 156/136 mmHg, in B mmHg

we use transducers which produce a sound field slightly broader than the diameter of the vessel and the sound field has approximately constant intensity across its middle part

Table 1 *Comparison between electromagnetic flow measurements and doppler velocity measurements for individual heart beats in one measurement series*

The comparison was performed on the common carotid artery for every third heartbeat. The table illustrates the typical degree of scatter between different cardiac cycles in a steady state situation

Observation	EM flow (ml/min)	Scale factor	Doppler flow (ml/min)	Calculated radius (mm)
1	219 1	13 7	210.3	1 45
2	216.3	13 8	210 1	1 46
3	216.3	13 9	211 6	1 46
4	234.3	14 1	234 7	1 47
5	235.3	14.3	228 6	1 48
6	232.3	14.3	223 1	1 48
7	219.2	14 0	216 0	1 47
8	214 7	14 0	211 9	1 47
9	216 6	14 1	208.2	1 47
10	199.9	13 9	190 4	1 46
11	195.3	13 6	182 0	1 45
12	196.3	13 6	178.3	1 45
13	185.5	13 1	176.5	1 42
14	162.3	11.5	154 4	1.33
15	197.9	13 4	186.5	1.43
Median				1 46
Confidence interval with confidence probability 0 95				[1 45–1 47]

Only two transducers have been used in the pr investigation one 6 MHz probe and one 1.5 MHz Fig. 1 shows the intensity distribution across the beam for various selected distances from the probe face for these two transducers The intensity distr were measured by means of a small ceramic crystal w was moved in different positions relative to the abov probes in a water tank

The electromagnetic flowmeter

A standard electromagnetic blood flow meter wa (type 372 Nycotron A/S Norway) Care was ta ensure a close fit of the transducers around the re vessel without compressing it Since drift of the ze voltage may be a problem with electromagnetic fl ters this voltage was determined repeatedly durin experiment by total compression of the artery Th transducers were calibrated in an in vitro system before and after the experiments.

Comput procedures

The doppler frequency Δf caused by blood cells with velocity is given by

$$\Delta f = \frac{2f_o \quad \cos\theta}{c}$$

where f_o is the emitted sound frequency (in one inst either 6 or 1.5 MHz) θ is the angle between the d of the sound beam and the blood velocity and c velocity of sound in the tissue (approximately 1 5 This formula is of course equally true if both Δf a substituted by the corresponding mean doppler fre $\overline{\Delta f}$ and mean blood velocity $\bar{v}$ across the vessel lu addition the instantaneous blood flow Q is given b

$$Q = A\bar{v}$$

imulatıon of gastric acid secretion and suppression ᴲVIP like immunoreactivity by bombesin the Atlantic codfish *Gadus morhua*

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HOLSTEIN B. & HUMPHREY C.S.. Stimulation of gastric acid secretion and suppression of VIP-like immunoreactivity by bombesin in the Atlantic codfish, Gadus morhua. Acta Physiol Scand 1980, 109 217 223. Received 26 Oct. 1979 ISSN 0001-6772. Department of Zoophysiology University of Göteborg Göteborg, Sweden and Department of Surgery Massachusetts General Hospital Boston, Mass. 02114, USA.

Cods were equipped with cannulae for drainage of the stomach and for separate perfusion of the stomach (pure sea-water) and intestine (diluted sea-water). Acidity and volume of gastric effluence were measured. Plasma immunoreactive gastrin and vasoactive intestinal polypeptide (VIP) were assayed in some experiments. The high rate of "basal" acid secretion was further elevated by m. administration of bombesin, but not by pentagastrin. Exogenous VIP inhibited acid secretion. Following 5 h of bombesin infusion, plasma gastrin-IR was unaffected while VIP IR was depressed compared to saline-treated controls. The possibility that bombesin stimulates acid secretion by inhibiting VIP-release is discussed

Key words Bombesin, gastric acid secretion, gastrin, fish physiology VIP radioimmunoassay

nbesin, a tetradecapeptide isolated from frogs n (Anastasi et al 1971) stimulates gastric acid retion in man, cat and dog (Bertaccini et al. 1973 4 Erspamer & Melchiorri 1975), but not in the (Bertaccini et al. 1973) The effect is mediated the release of antral gastrin (Basso et al. 1974 '5 Bertaccini et al. 1974 Impicciatore et al 1973 74) There is evidence for the presence of bombe-like material in the gastrointestinal tract of frog, ı, and man (Polak et al 1976 Walsh & Holmest 1976)

In codfish, rendered water-deficient by pyloric ation, basal acid secretion is low or absent olstein 1979) Histamine and carbachol are ective secretagogues but no response was cited by i m pentagastrin (Holstein 1975 1976) bombesin (Holstein, unpublished) The finding at cods which have been water-repleted by intestinal perfusion with diluted sea-water exhibit gh rates of basal" acid secretion (Holstein 79) called for reinvestigation of pentagastrin d bombesin effects on gastric acid secretion In a rallel, and intentionally separate investigation, we examined the possible involvement of vasoactive intestinal polypeptide (VIP) in the regulation of codfish acid secretion. Some results from that work is included in the present report.

MATERIALS AND METHODS

Animals Codfish of both sexes, weighing 365–620 g were used. Before experiments they were kept in storage aquaria with recirculating, chilled (10°C) sea-water for at least one week. They were not fed.

Surgery The animals were surgically prepared for the separate perfusion of the stomach and intestine and for drainage of the stomach. The technique has been described in detail (Holstein 1979)· the cannulae positioned were C1 C2 and C3

Postsurgical treatment Immediately following recovery from anaesthesia (MS-222, Sandoz) the fishes were transferred to smaller tanks and perfusion of the intestine (33% SW[a]) and stomach (100% SW[a]) containing

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[a] 100% SW indicates pure sea-water 33% SW is mixture of one part sea-water and two parts distilled water

own right Our results so far have however shown that the scale factors correspond to internal diameters of the right order of magnitude

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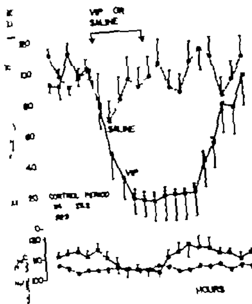
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1 Effect of saline (=8) and 20 μg/kg h VIP (n=5) gastric acid secretion and volume output.

in alkaline. This antibody shows no evidence of cross-tivity with other peptides. The lower limit of detection 5 pg/ml. All samples from any one experiment were ayed together

Statistics

results are presented as mean ± S.E. or as mean and ± E. For comparison of means, the Mann-Whitney test or the Kruskal-Wallis one-way analysis of variance (Siegel 1956) were used. The level of significance was set 05 (two-tailed).

RESULTS

Secretory effect

1. Pentagastrin The pentapeptide was administered at 2 and 20 μg/kg h (Fig 1). In both series during the control period, the secretory rate was increasing. During the 1st treatment h (lower dose) a value significantly different from the control was obtained, and in the late post-treatment period (higher dose) depressed acid secretion was noted. The result presents no convincing evidence that the pentapeptide, at the doses employed, possesses any stimulatory effect.

2. *Bombesin* The tetradecapeptide was administered at 0.5 and 2.5 μg/kg h. Both doses increased the secretory rate (Fig 2). With the lower dose stimulation was encountered in all the 10 individuals, while with the higher dose 10 were stimulated and 2 inhibited

3. *VIP* 20 μg/kg h was administered to 5 animals. Acid secretion rapidly decreased and remained depressed for 7 h following the outset of the peptide (Fig. 3).

Plasma gastrin-IR and VIP IR

The effect of 5 h VIP or bombesin administration on plasma levels of gastrin-IR and VIP IR appears in Table 1. The secretory effects confirming the above, are shown in Fig. 4. VIP injected at 20 μg/kg h increased the plasma level of VIP IR to about 3 times that of saline-treated controls, and tended to decrease the level of gastrin-IR. The later effect was not statistically significant. Bombesin (2.5 μg/kg h) depressed the level of VIP IR but did not affect gastrin-IR. There was no difference (by analysis of variance) among the % b wt. values (Table 1). These values indicate that the fishes, at the time of sacrifice were in proper water balance (Holstein 1979*a*)

Volume effects

Gastric volume outputs appear in the lower panels of the figures and are expressed as percentages of

Table 1. *Final body weight and plasma concentrations of IR-gastrin and IR VIP in fishes treated with saline, VIP (20 μg/kg h) or bombesin (2.5 μg/kg h) during 5 h*

n animals in each group. Gastric acid secretion is shown in Fig. 4. The *P*-values are vs. the saline series (Mann-Whitney U-test)

Treatment	% b.wt.	IR-gastrin (pg/ml)	IR-VIP (pg/ml)
Saline	101.5±0.5	35.4±2.9	418.6±57.8
VIP	101.1±0.5	27.6±3.2 (*P*=0.15 NS)	1 366.0±79.7 (*P*=0.008)
Bombesin	102.4±0.6	34.8±4.0 (*P*=0.84 NS)	173.6±30.9 (*P*=0.008)

Body weight at the time of sacrifice expressed as percentage of the presurgical b.wt.

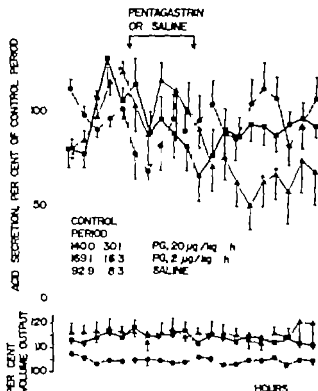


Fig 1 Effect of saline and two doses of pentagastrin 2 and 20 µg/kg h on gastric acid secretion and gastric volume outflow The series comprised 8 10 and 5 animals respectively In this and the following figures the mean secretory rates (µmol H /kg h ± S E.) during the respective control periods are given Asterisks denote a significant difference ($P<0\,05$ $P<0\,01$) between saline-treated and hormone-treated animals.

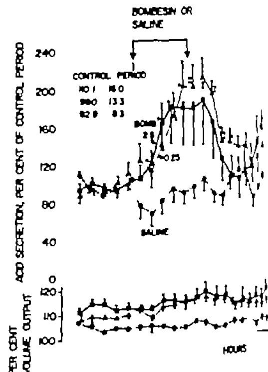


Fig 2 Effect of saline and two doses of bombesin ... and 2.5 µg/kg h. The series comprised 8, 10, ... animals, respectively

100 mg/l phenol red as a volume marker started Perfusion rate for both stomach and intestine was about 8.5 ml/h but varied between pumps and experiments The actual rate was recorded following each experiment Gastric outflow was collected in 1 h fractions The fishes were left without further treatment for at least 20 h

Experimental protocol

1 *Secretory effects of exogenous peptides* Pentagastrin (Peptavlon, ICI) bombesin (a generous gift from Dr R de Castiglione Farmitalia Milano) VIP (natural porcine VIP generously supplied by Prof V Mutt GIH Laboratory Karolinska Institutet Stockholm) or saline (1 05 % NaCl) was injected for 5 h Gastric acid secretion was assayed for 5 h before during and for 10 h following treatments. All injections were by the i.m. route at a rate of 0 62 ml/h

2. *Effect of bombesin and VIP on plasma gastrin- and VIP-immunoreactivity* Following 5 h of bombesin VIP or saline administration the fishes were removed from the aquaria, stunned with a blow on the head and 5 ml of blood drawn from the caudal vein The syringe contained 5000 KIU aprotinin (Sigma) and 200 IE heparin (Vitrum) The plasma was separated lyophilized and sent to Boston for peptide analyses. Gastric acid secretion was assayed for 5 h before and during treatments

Analyses and presentation of the data

1 *Gastric secretion* The samples were analysed ... acidity volume and phenol red (PR) concentration ... described earlier (Holstein 1979*a*) From the amount ... PR infused and the amount of PR in the effluent ... the recovery was calculated and the outflow ... accordingly corrected The acid content was calculated from acidity and corrected volume and expressed as ... H /kg h In calculating this, the presurgical ... used In the figures acid secretion has been ... that the mean acid secretion during the 5 h (control p... preceding change of the experimental situation ... 100 % Gastric volume output is given as a perc... of the hourly infused volume

2. *Radioimmunoassays*

VIP Plasma VIP concentration was measured by a ... tive RIA described previously (Ebeid et al 197...) ... antibody raised in rabbits against highly purified ... VIP conjugated to bovine serum albumin Highly ... and synthetic VIP produced equal displacement ... labelled VIP from the antibody The assay had a ... limit of detection of 7.5 pg/ml All samples from ... experiment were assayed together In triplicate ... with determinations of nonspecific binding in the ... of antibody No cross reactivity has been detected ... a number of peptides in concentrations up to 10 ... including the related peptides secretin, glucagon ...

Gastrin Gastrin was assayed in a previously de... RIA (Dent et al 1972) using antibody prepared in ... against synthetic human gastrin 17 conjugated to ...

glucagon-like immunoreactivity is present in same cell (see Falkmer & Östberg 1976).) Al- ·gh the common C-terminal sequence of gastrin CCK appears to be present, this study failed to in evidence that this sequence alone (i.e. penta- ·rin) is sufficient for the stimulation of acid etion in the water-repleted cod. Therefore the ·ious failure of pentagastrin to induce acid ·etion in water-depleted cods (Holstein 1975) ·s not solely due to the dehydration. Dehydration ·d, however explain the inefficiency of bom- · in water-depleted cods (Holstein, unpub- ·ed). The exact mechanism remains to be worked

·imulation of acid secretion by bombesin in ·mals and man and the concomitant release of ·al gastrin (Basso et al 1974 1975 Bertaccini et · 1973 1974 Impicciatore et al. 1973 1974), to- ·ther with the finding of bombesin-like material in ·mal species (Polak et al. 1976 1978 Walsh & ·mquist 1976) suggest that a bombesin-like ·tide may play a role in the regulation of gastrin ·ase (Erspamer & Melchiorri 1975). In concert · this suggestion is the evidence presented by ·mpson et al. (1976) and by Llanos et al. (1977) · gastrin release following intestinal feeding may ·olve an endogenous bombesin-like peptide

·n the codfish, too bombesin stimulated acid ·retion but it did not elevate the level of plasma ·trin-IR. Thus, the mechanism appears not to · on the release of gastrin and therefore is dif ·nt from that in mammals The uncertainty in- ·ent in this conclusion, due to incompletely char ·rised antigen (codfish gastrin") and antibody ·uld again be stressed. The conclusion seems ·ever supported by the inefficiency of penta- ·trin. Although direct activation by bombesin of · acid secretory cell seems excluded in mammals ·sso et al. 1975 Impicciatore et al 1973), such ·echanism cannot be excluded in the codfish ·wever it is also possible that bombesin releases ·imulatory principle not detected by the gastrin ·ay Such a mechanism was suggested by Hir ·owitz & Gibson (1978) who wrote: "Bombesin ·s potent gastric stimulus whose action is only ·tially explained by the measured gastrin re- ·se Release of CCK by bombesin has been sug- ·ted (Konturek et al. 1976*b*) and there is evi- ·ce for release also of pancreatic polypeptide ·) (Taylor et al 1977) and both peptides stimu- · mammalian acid secretion in the absence of gastrin (Grossman 1971 Lin et al. 1977). The octa- peptide of CCK inhibits basal" secretion in the cod (Holstein, unpublished) making CCK less attractive as the stimulatory principle and PP has not been tested for secretory effects in fish, though its pres- ence in several teleost species has been demon- strated (Van Noorden & Patent 1978).

Since the secretion of acid reflects the sum of stimulatory and inhibitory agents impinging on the secretory cell, there is also the *third possibility that* bombesin increases acid secretion by suppression of an inhibitor We found a significant decrease in plasma VIP IR and therefore concerning the cod, consider the third alternative likely The increase in a gastrin-like peptide not detected by the gastrin assay can, however not be excluded The sugges- tion that bombesin stimulates acid secretion by in- hibition of VIP release is tempting, but no signifi- cant correlation was found between plasma VIP-IR levels and acid secretion (in absolute terms or in per cent of control period) in the bombesin-treated animals. The importance of this, however is dif ficult to evaluate as long as the individual compo- nents driving the basal" secretion are qualitatively and quantitatively unknown. Pertinent is the ob- servation that feeding results in an initial inhibition of plasma VIP IR in the dogfish shark (Humphrey unpublished)

Volume output Theoretically gastric volume output includes (i) infused volume (ii) ingested volume and (iii) gastric volume secretion. Perfusion of the intestine, with pure or diluted sea-water at the rate presently used, prevents drinking (Holstein 1979 1979*b*) During intestinal acidification, how ever drinking occurs and mean volume output amounts to above 200% (Holstein 1979*b*). Also intestinal perfusion with isosmotic glucose (Hol- stein & Cederberg 1980) increases volume output (to a mean of 160%)· this increase probably also being an effect of oral water ingestion. The volume changes encountered in the present experiments were of much less magnitude and most likely reflect changes in gastric volume secretion The most ob- vious deviation from the covariation between acid secretory rate and volume output occurs in the VIP postinjection period (Fig. 3), and could indicate that acid secretion can change independently from volume secretion. Effects on water ingestion seem unlikely but are not xcluded and may contribute.

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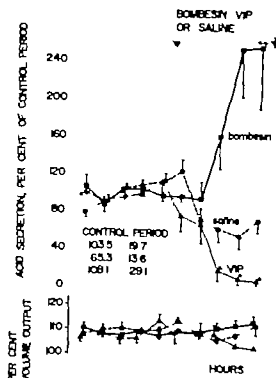


Fig 4 Effect of saline VIP (20 μg/kg h) and bombesin (2.5 μg/kg h) 5 animals in each group Following the 5th treatment h blood samples for hormone assay were collected (Table 1)

the gastric perfusion rate Despite this normalization the outputs varied between the different series and were therefore not statistically compared Generally the change in volume output mirrors the change in acid secretory rate Thus no obvious change was noted during pentagastrin infusion (Fig 1) while during VIP administration volume output decreased (Figs 3 4) However following termination of VIP volume output recovered more rapidly than acid secretion (Fig. 3) Bombesin which stimulated acid secretion increased volume output (Figs 2 4) the increase being most obvious during the lower dose

DISCUSSION

Acid secretion VIP a polypeptide of 28 amino acids isolated from hog small intestine (Said & Mutt 1970) has a wide spectrum of biological actions (Said 1975) Mammalian gastric acid secretion stimulated by food (Villar et al 1975 1976) histamine (Konturek et al 1976*a*) pentagastrin (Makhlouf et al 1978 Schorr et al 1974) and gastrin (Villar et al 1975) is inhibited while basal secretion is not (Villar et al 1976) VIP also inhibits stimulated gastrin release but does not affect basal le[vels] of gastrin (Villar et al 1975) It has been sugg[ested] that blockade of gastrin at the parietal cell le[vel is] the major mechanism by which VIP inhibits [...] gastric acid secretion (Konturek et al. 1976*a*) [The] present results indicate that a VIP-like m[aterial] circulates in the codfish The basal plasm[a con]centrations are similar to those measured in dogfish shark (Humphrey unpublished) but [low] when compared to man dog, and rat (<1% [...] with this assay) Previously by bioassay and [...] evidence has been presented for the occurre[nce of] similar material in extracts from cod and [...] testine (Dockray 1974 1975 1976). The pre[sent] work also seems to indicate that "basal" acid se[cre]tion is depressed by VIP but the nature of t[his] secretion though arbitrarily designated "basal" [is] incompletely characterized The secretion is de[pendent] on an intact vagal supply (Holstein & [...]derberg 1980) but this does not exclude the in[]volvement of a stimulating hormone Gastrin [...] was in fact detected in codfish plasma but the [fai]lure of pentagastrin to stimulate the acid secr[etion] makes it difficult to ascribe part of the "ba[sal"] secretion to this immunoreactivity The p[lasma] level was not significantly affected by exog[enous] VIP in an amount elevating the plasma VIP [to] about 300% of the control level (Table 1). Th[is is] in accordance with VIP effects on basal gast[rin in] the dog (Villar et al 1976) The findings must h[ow]ever be carefully interpreted because i) the [se]quence of the gastrin molecule recognized b[y the] antibody used is undetermined and ii) the cod "gastrin" is not wholly characterised (Larsson & Rehfeld 1977) The mechanism of action of VIP [in] the cod whether on the oxynticopeptic cell [or] or on the release of a stimulating peptide is [still] undetermined

Using an antibody specific for the common [COOH] terminus of gastrin and CCK Larsson & Reh[feld] (1977) found endocrine cells in the gut of mem[bers] of all vertebrate classes including the cod [An] antiserum directed against the NH_2-terminal p[art of] gastrin demonstrated endocrine cells in the hi[gher] vertebrates but failed to do so in frog and [...] In these later animals the cells reactive with COOH terminus specific antibody also reacted [with] an antiserum specific for the sequence [...] CCK 33 The authors questioned the existence [in] the lower vertebrates of gastrin and CCK as se[pa]rate hormones (In cyclo tomes gastrin/CCK [...]

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sons Stiftelse och Harald och Greta Jeanssons Stiftelse Wilhelm och Martina Lundgrens Vetenskapsfond and from Långmanska Kulturfonden The skilful technical assistance from Mrs Inga-Maj Örbom is gratefully acknowledged We thank Mr Ingmar Hakemar for supplying fish and Dr Josef E. Fischer for the facilities and encouragement to perform the radioimmunoassays

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Physiol Scand 1980 109 225–229

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HOLMBERG E. & WALDECK, B The effect of insulin on skeletal muscle contractions and its relation to the effect produced by β-adrenoceptor stimulation. Acta Physiol Scand 1980 109 225–229 Received 26 Oct. 1979 ISSN 0001-6772. Research and Development Laboratories, AB Draco, Lund, Sweden.

The soleus, slow-contracting, and the extensor digitorum longus (EDL), a fast-contracting muscle, from the guinea-pig were prepared for measurement of isometric contractions in vitro. Insulin, 2.5–55 mu/ml caused dose-dependent depression of twitches and subtetanic concentrations of the soleus muscle similar to and additive with that produced by the β_2-adrenoceptor agonist, terbutaline The effect of terbutaline but not that of insulin was blocked by propranolol. Insulin had no apparent effect on the contractions of the EDL, whereas terbutaline increased the force of contraction. When depressed by KCl, however insulin partially restored the twitch tension in both muscles. The possible role of effects on the N –K transport is discussed.

Key ords. Insulin β-adrenoceptor terbutaline skeletal muscle, contraction, guinea-pig

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To test the possibility of causal relationship tween the stimulation of the Na K transport d the changes in contraction we have studied the fect of insulin. This hormone has long been own to affect ionic fluxes in skeletal muscle reese 1964 Zierler 1966). More recent studies show that the active transport of Na and K ions through the cell membrane of the muscle is stimulated (Clausen & Kohn 1977) presumably by a mechanism unrelated to changes in c AMP (Flatman & Clausen 1979*b*).

Further we have compared the effect of insulin on skeletal muscle contractions with that of terbutaline a β_2-selective adrenoceptor agonist (Bergman Persson & Wetterlin 1969 Persson & Olsson 1970) Measurements have been made on isolated preparations of the soleus a slow-contracting and the extensor digitorum longus (EDL), a fast-contracting muscle from the guinea-pig (Ariano Armstrong & Edgerton 1973)

METHODS

Male guinea-pigs, about 200 g, were used. The soleus and the EDL muscles were dissected out under pentobarbitone anaesthesia and were mounted in organ baths containing oxygenated Krebs solution at 37° Subtetanic contractions were evoked by transmural field stimulation. Trains of supramaximal pulses of 0.5 ms duration were delivered every 20 s from Grass S 88 stimulator For

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To test the possibility of a causal relationship tween the stimulation of the N K transport d the changes in contraction we have studied the fect of insulin. This hormone has long been own to affect ionic fluxes in skeletal muscle reese 1964 Zierler 1966) More recent studies show that the active transport of Na and K ions through the cell membrane of the muscle is stimulated (Clausen & Kohn 1977) presumably by a mechanism unrelated to changes in c AMP (Flatman & Clausen 1979*b*)

Further we have compared the effect of insulin on skeletal muscle contractions with that of terbutaline a β_2-selective adrenoceptor agonist (Bergman, Persson & Wetterlin 1969 Persson & Olsson 1970) Measurements have been made on isolated preparations of the soleus a slow-contracting and the extensor digitorum longus (EDL), a fast-contracting muscle from the guinea-pig (Ariano Armstrong & Edgerton 1973)

METHODS

Male guinea-pigs, about 200 g, were used. The soleus and the EDL muscles were dissected out under pentobarbitone anesthesia and were mounted in organ baths containing oxygenated Krebs solution at 37° Subtetanic contractions were evoked by transmural field stimulation. Trains of supramaximal pulses of 0.5 ms duration were delivered every 20 from Grass S 88 stimulator For

Subsidiary to AB Astra, Sweden.



Fig 1 The effects of insulin and terbutaline on the force of contraction of an isolated soleus muscle from guinea pig. Subtetanic contractions and twitches were measured alternately Terbutaline 2.3 μmol/l was added at the left arrow (TRB) After rinsing and recovery insulin 50 mu/ml was added (INS) the right arrow

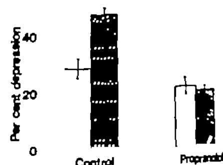


Fig 2 Combined effect of insulin and terbutaline and inhibition by propranolol on subtetanic contractions of isolated preparations of the guinea-pig soleus muscle. The open columns show the effect of 25 mu/ml insulin. The hatched columns show the effect after a further addition of 2.3 μmol/l terbutaline When present, propranolol 1 μmol/l was added 30 min before insulin. Mean SE of expts

the soleus the stimulus frequency was about 12 Hz and the train duration 1.5 s For the EDL these parameters were 40 Hz and 150 ms respectively Ten seconds after each train a single pulse was given to monitor the twitch tension of the muscles All measurements were made isometrically For more details see Waldeck (1976)

When stable contractions had been established the response to a supramaximum dose of terbutaline (2.3 μmol/l) was determined. The mean maximum depression ±S D of the soleus muscle was 32±6% The subtetanic contractions of the EDL muscles increased by 56±18% After a rinsing and recovery period the muscles were ready for use Details of the experiments are given in the results section

The following drugs were used Porcine insulin (Sigma) terbutaline sulphate (Draco) and propranolol (ICI) Solutions were made up in saline Statistical evaluation of the data was made by Student s *t*-test.

RESULTS

(a) *Pilot studies*

Insulin caused in the soleus muscle a depression of subtetanic contractions qualitatively similar to that produced by terbutaline (Fig 1) However the effect of insulin developed more slowly than that of terbutaline and the maximum effect appeared to be smaller In the EDL insulin up to 50 mu/ml had no

Table 1 *Effect of insulin on the force of subtetanic contractions and twitches of the isolated soleus muscle from the guinea-pig*

The data are the means ±S.E of 5 expts

Insulin (mu/ml)	Per cent depression: Subtetanic contractions	Twitches
2.5	7 6±1 7	_6±1 6
12	11 0±3 0	7 4±2.3
55	14.8±2 0	10.8±2 9

effect on the force of contraction but a slight depression which could not be distinguished from the usual slow deterioration of the preparation (cf Holmberg & Waldeck 1977)

In our pilot studies we also found that once the muscle had been exposed to insulin it was insensitive to further additions of the hormone, even after washing Therefore each muscle was used only once in the following experiments

(b) *Dose response relation for insulin*

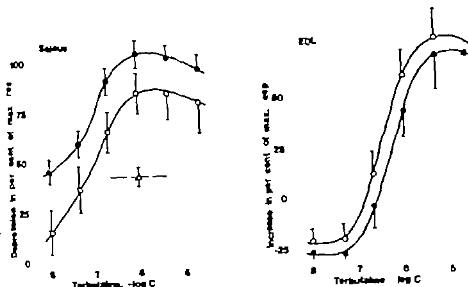
In the soleus muscle insulin from 2.5 to 55 mu/ml caused a dose-dependent depression of the subtetanic contractions as well as of the twitch tension ($P<0.05$) The effect on the subtetanic contractions tended to be greater than that on the twitches (Table 1)

The mean time ±S E. to reach half the effect produced by 55 mu/ml insulin was in 5 expts 4 8±0 3 min The corresponding figure for the maximum effect of terbutaline is of the order 1 min (Holmberg Svedinger & Waldeck 1979)

(c) *The effect of propranolol*

In the next experiment on soleus muscles insulin mu/ml was given and after about 15 min when the effect had developed terbutaline 2.3 μmol/l was added to the bath In half of the expts propranolol 0 3 μmol/l had been given 30 min before insulin The result is shown in Fig 2.

Insulin caused a depression of the force of subtetanic contractions by 20–30% Terbutaline caused a further depres contractions



f. Cumulative dose-response curves for terbutaline in the presence and in the absence of insulin. Insulin, 25 mu/ml, given to preparations of soleus and EDL muscles. After about 30 min terbutaline was added cumulatively (closed ...s). Control muscles without insulin were run in parallel (open circles). The effect of insulin alone on the soleus ...le is denoted by a triangle. Shown are the means ± S.E. of 7–8 expts. Ordinate: Changes in the force of subtetanic ...ractions in per cent of the maximum response to a test dose of terbutaline (see Methods).

...ct being almost doubled ($P<0.005$) Propranolol ...not affect the insulin-induced depression but ...bited completely that caused by terbutaline

Interaction between insulin and terbutaline

...lin, 25 mu/ml was given to preparations of ...us and EDL muscles. After about 30 min, when ...effect of insulin had developed, terbutaline was ...ed cumulatively. Control muscles without insu... were run in parallel. The effects were calculated ...vidually in per cent of the maximum response to ...µmol/l of terbutaline. The changes refer to the ...e at the time for the addition of insulin.

As in the previous expts. insulin caused a depres...n of the subtetanic contractions of the soleus ...scle (Fig. 3). Terbutaline added to this effect in ...se-dependent manner and at 0.22 µmol/l the ...mbined effect was twice that elicited by insulin ...ne ($P<0.001$) but less than that produced by ...butaline at that dose level ($P<0.05$). Moreover ...e maximum depression obtained by terbutaline ...peared to be greater in the presence than in the ...sence of insulin but this difference did not be... me statistically significant.

There was no apparent effect of insulin on the ...ntractions of the EDL muscle. Nor did insulin ...ange the response to terbutaline in this prepara...on (Fig. 3). However during the time for the preincubation with insulin both the treated and the control muscles lost in force some 10% (cf Holmberg & Waldeck 1977). This is the reason why the cumulative dose-response curves for terbutaline start at a point below zero.

(c) *Effect of insulin on muscle depressed by KCl*

In some experiments on soleus and EDL muscles KCl was added to the bath until the contractions of the muscles were depressed by about 50%. This occurred at a final K-concentration of 12–13 mmol/l usually higher for the EDL than for the soleus. Then insulin, 25 mu/ml was added.

In all expts. the progress of the depression caused by excess K was broken and reversed by insulin in a few minutes. The increase in the force of contraction calculated in per cent from the bottom of the dip ranged from 9–53% in 5 expts. on the soleus. In 4 expts. on the EDL the increase ranged from 15–4.% The increase in the force of contraction of the EDL was significantly different from zero ($P<0.05$). Records from 2 of the expts. are shown in Fig. 4.

DISCUSSION

Insulin caused a dose-dependent depression of the subtetanic contractions of the soleus muscle similar

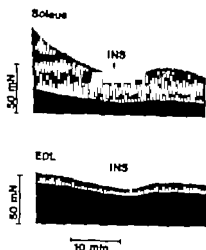


Fig 4 Partial reversal by insulin of the depression of the force of contraction caused by excess K in the medium Subtetanic contractions and twitches were measured alternately The [K] was 1? and 14 mmol/l for the soleus and the EDL, respectively The addition of 25 mu/ml insulin is indicated by an arrow

to that produced by terbutaline It is pertinent that this effect occurred in the same dose range as the stimulation by insulin of the Na K transport in the rat soleus in vitro (Clausen & Kohn 1977) The failure of propranolol to inhibit the effect of insulin indicates that this hormone acts on a site beyond the β adrenoceptor

In a previous paper (Holmberg & Waldeck 1980) we have pointed out the similarity between the dose response curves for the effect of β-adrenoceptor agonists on the force of contraction (Waldeck 1977) and those on the Na K transport (Clausen & Flatman 1977) in the soleus muscle Further the effects of insulin and terbutaline on subtetanic contractions are additive (the present data) as are the effects of insulin and adrenaline on the Na K transport (Flatman & Clausen 1979*a* and *b*) Moreover the observation that the effects of insulin on the Na K transport are less pronounced and slower in onset than those of adrenaline (Flatman & Clausen 1979*a* and *b*) also parallels our functional studies on insulin and terbutaline

These data suggest that there is a causal relationship between the stimulation of the Na K pump of the cell membrane and the depression of the contractions of the soleus muscle The stimulation by β-adrenoceptor agonists and insulin of the Na K transport in the soleus muscle is blocked by ouabain (Clausen & Flatman 1977 Clausen & Kohn 1977) Experiments to block by ouabain the effects of the agents on the force of contraction are more difficult to interpret because ouabain per se affects the contractile force of the muscle (Ta... 1973 Holmberg & Waldeck 1980 and unp... data) However the view that changes in the ... Na balance may control the intracellular m... ments of Ca² which regulate the contr... machinery of the muscles is supported by ... evidence (Tashiro 1973 Bowman 1980, Holmberg & Waldeck 1980)

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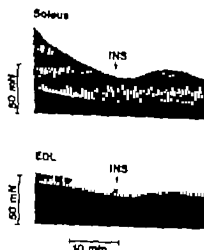


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mulation of HCO_3^- transport by gastric inhibitory peptide P) in proximal duodenum of the bullfrog

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mal duodenum isolated from the bullfrog ely transports HCO_3^- into the lumen, a pro- which is inhibited by acetazolamide and methacin and stimulated by prostaglandins ström 1979 1980a) Duodenal mucosa in this al is devoid of Brunner glands, indicating that e HCO_3^- transport is a property of the surface lial cells. HCO_3^- is secreted by duodenal epi- um also in the dog in vivo and acetazolamide eases the rate of loss of intraduodenally instil- acid in this species (Harmon Woods & Gurll). These results suggest that epithelial HCO_3^- sport contributes to duodenal disposal of lumi- acid. The ability of gastric inhibitory peptide) to inhibit gastric emptying and acid secretion rea Polak & Bloom 1977) should decrease de- y of H ions into the duodenum. The latter may ndicative of an ulceroprotective function and e it of interest to examine the effects of GIP on denal epithelial HCO_3^- transport.

ethods External muscle layers were removed the proximal duodenum of the bullfrog (*Rana iberiana*) and the resulting mucosal cylinder osed length 12 mm, mounted between two glass is connected to a reservoir An unbuffered mal bathing solution (5 ml) was circulated by a lift of 100% O and HCO_3 transport titrated at 7.40 with 5 mM HCl under control from a pH system (Radiometer Copenhagen, Denmark) nutrient (serosal) side solution (250 ml) was fered at pH 7.20 with 0.8 mM phosphate and 8 mM HCO_3 and contained glucose (2 mM). This ution was gassed with mixture of 95% O and CO Both solutions contained Na 102.4 K Ca 1.8, Mg 0.8 and Cl^- 91.4 mM. Mannitol 1.50 were added to the luminal solution to ieve isotonicity (221.8 mosM). GIP was added to serosal side in small volume (50 µl for 10^{-9} M) nsepithelial lectrical potential difference was measured in two matched calomel electrodes Effects of GIP on HCO_3^- transport were examined also in gastric fundic mucosa from *Rana temporaria* in which H secretion was abolished by pretreatment with 10^{-4} M Metiamide (Flemström 1977) Compositions of the bathing solutions and titration of HCO_3 transport were as described for duodenum Purified porcine GIP was a kind gift from Professor Viktor Mutt Department of Biochemistry II Karolinska Institutet, Stockholm, Sweden.

Results In untreated duodenum there was a small continual rise in the electrical potential difference and a tendency for the rate of HCO_3^- transport to decline over a period of 4 h (Fig. 1). Administration of GIP (10^{-10} M) caused a significant increase ($0.01<P<0.02$) in the rate of HCO_3^- transport compared with that in the preceding control period. Addition of 10^{-9} M GIP resulted in a further increase ($P<0.01$ compared with the rate at 10^{-10} M) and in 3 expts. a still further rise of 0.05–0.10 µeq/h occurred when the concentration was increased to 10^{-8} M. The slight variations in transepithelial electrical potential difference in GIP treated duodenum were similar to those observed in untreated controls. In Metiamide-treated fundic mucosae HCO_3^- transport decreased from 0.29±0.03 to 0.20±0.03 µeq/h cm² during 90 min exposure to 10^{-9} M GIP and to 0.16±0.03 µeq/h, cm² during a further 90 min exposure to 10^{-8} M GIP (means ± S.E. =7) These changes were somewhat greater than the slight decline in HCO_3^- rate occurring in control mucosae

Discussion The present results demonstrate that duodenal surface epithelial transport of HCO_3 is stimulated not only by prostaglandins with lcero-protective properties but also by the hormone GIP A significant increase in transport rat occurred with 10^{-10} M GIP while in this same duodenal preparation, the minimal effective concentrations of

cts of castration and testosterone substitution on y composition and muscle metabolism in rats

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KROTKIEWSKI M KRAL, J G & KARLSSON J Effects of castration and testosterone substitution on body composition and muscle metabolism in rats. Acta Physiol Scand 1980 109 233–237 Received 20 Sept. 1979 ISSN 0001-6772. Department of Rehabilitation Medicine II Department of Surgery II Sahlgrens Hospital University of Göteborg, Sweden and GIH Stockholm, Sweden

Male weanling Wistar rats were castrated or sham-operated and followed for 12 weeks without substitution or with large (2 mg 14 days) or small (0.2 mg 14 days) intramuscular dose of testosterone enantate. Castration without substitution was associated with lower body weight and smaller fat cell sizes in different adipose tissue depots The epididymal and caudal subcutaneous depots were the most sensitive to castration. The percentage of fast-twitch high-oxidative (type II A) muscle fibers decreased in the non-substituted castrated animals There was decrease in phosphorylase and lactate dehydrogenase activities in the white portion of the gastrocnemius muscle of the castrates. These changes ere reversed by the large dose of testosterone Removal of testosterone by castration thus seems to "feminize" male rats with respect to body composition and muscle metabolism.

Key ord Castration testosterone, body composition fat cells muscle fibers, muscle enzymes

st species males have a large muscle mass and antly greater strength than females This rence between sexes is not apparent until puat which time changes occu in work per ance and muscle metabolism (Eriksson 1973). Differences in the percentage distribution and al area of muscle fibers have been found in Gutman et al 1969) and man (Nygaard et al Edstrom et al 1969). The findings have been ght to depend on differences in physical acti ather than on hormonal effects (Saltin et al.). Since puberty is characterized by increased tion of gonadal hormones it seems plausible xplain the sex differences in body composition muscle metabolism as being due to these hor es. The aim of this study was to elucidate the ts of castration and substitution of testosterone body weight and composition and skeletal cle composition and enzyme activities in ing male rats.

male W tar SPF rats with mean
ac groups in Macrolon

cages for one week. They were maintained in temperature controlled room (21°C) with stable humidity (50–60 %), fed ad libitum with commercially available rat pellet diet (EWOS Södertalje Sweden) and had free access to tap water

The rats were randomly assigned to 4 groups of 10 rats (I–IV). After being weighed 3 of the groups (I–III) were castrated by simple orchiectomy sparing the epididymis and its fat pad (Kral & Tisell 1976), while one group (IV) was sham operated using the same scrotal incision and handling the testes to simulate the operative trauma. The operations were performed in pentobarbital anesthesia (Mebumal ACO S eden). Because of pulmonary infection one rat was excluded from the sham operated group IV

The rats ere allowed to recover for one week before starting the series of bi-weekly intramuscular injections of testosterone enantate (Testoviron-Depot Schering A G Berlin W Germany), or only the same volume of the vehicle sesam oil according to the following plan

C trated rat

Group I large dose: mg of testosterone every 14 days
Group II small dose: 0.2 mg of testosterone every 14 days
Group III sham injection. 0 1 ml of sesam oil e ery 14 days

Sham-operated rat

Group IV sham injection: 0 1 ml of sesam oil every 14 days

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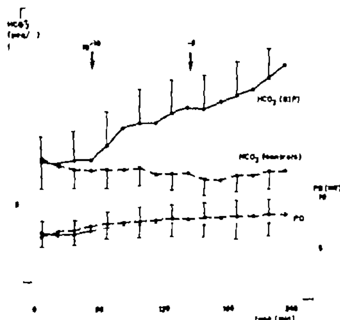


Fig 1 GIP was added as indicated to the nutrient (serosal) side of isolated duodenal mucosae Means ± S E of the rate of HCO_3 transport and transepithelial electrical potential difference (PD) in GIP-treated mucosae ($n=7$) and untreated controls ($n=9$) are shown

E type prostaglandins was 10 M (Flemström 1980a) However the rate of HCO_3 transport rose more slowly after GIP compared with the prosta glandins and the maximal rate appears somewhat lower This may reflect different modes of action and/or slower penetration of the polypeptide from the nutrient solution to HCO_3 transporting cells in the mucosa

Gastric mucosa in vitro also displays an active HCO transport and secretion of HCO_3 has been demonstrated in guinea pig and dog stomach in vivo The properties of surface epithelial transport of HCO in stomach and duodenum are similar but not identical (Flemström 1980b) Evidence obtained in the dog suggests that gastric HCO transport is controlled by a humoral mechanism since acid in stilled into the main stomach stimulated HCO se cretion from a denervated Heidenhain pouch (cf Allen & Garner 1980) The inhibitory action of GIP on gastric HCO_3 transport in vitro makes unlikely that this hormone is involved in humoral stimula tion of HCO_3 secretion in the stomach in vivo Humoral control of duodenal surface epithelial HCO_3 transport in vivo has not been studied and further work is required to evaluate the possible function of GIP as a mediator of duodenal protec tion The abnormally high serum concentrations of GIP in some patients with duodenal ulceration (Cataland et al 1977) may possibly reflect de creased feedback inhibition of hormone release.

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KROTKIEWSKI M KRAL, J G. & KARLSSON J Effects of castration and testosterone substitution on body composition and muscle metabolism in rats Acta Physiol Scand 1980 109 233-237 Received 20 Sept 1979 ISSN 0001-6772. Department of Rehabilitation Medicine II Department of Surgery II Sahlgrens Hospital University of Göteborg, S eden and GIH Stockholm, S eden

Male ealing Wistar rats were castrated or sham-operated and followed for [illegible] weeks without substitution or with large (2 mg 14 days) or small (0.[illegible] mg 14 days) intramuscular dose of testosterone enantate. Castration without substitution wa associated with lower body weight and smaller fat cell sizes in different adipose tissue depots The epididymal and caudal subcutaneous depot were the most sensitive to castration. The percentage of fast-twitch high-oxidativ (type IIA) muscle fibers decreased in the non-substituted castrated animals There was decrease in phosphorylase and lactate dehydrogenase ac tivities in the white portion of the gastrocnemius muscle of the castrates. These changes were reversed by the large dose of testosterone Removal of testosterone by castration thus seems to "feminize male rats with respect to body composition and muscle metabolism.

Key words Castration, testosterone body composition, fat cells, muscle fibers, muscle enzymes

[In m]ost species males have a large muscle mass and [con]stantly greater strength than females. This [diff]erence between sexes is not apparent until pu[bert]y at hich time changes occur in work per[form]ance and muscle metabolism (Eriksson 1973) [Sex] differences in the percentage distribution and [sur]face area of muscle fibers have been found in [rat]s (Gutman et al. 1969) and man (Nygaard et al [197]6, Edström et al. 1969). The findings have been [thou]ght to depend on differences in physical activ[ity] rather than on hormonal effect (Saltin et al [19]77) Since puberty is characterized by increased [secre]tion of gonadal hormones, it seems plausible [to] explain the sex differences in body composition [an]d muscle metabolism as being due to these hor[mo]nes The aim of this study was to elucidate the [ef]fects of castration and substitution of testosterone [o]n body weight and composition and skeletal [mu]scle composition and enzyme act vities in [gro]wing male rats.

[M]ETHODS

[Aft]er weaning, 40 male Wistar SPF rats with mean [...] 64 g were acclimatized in groups in Macrolon cages for one week. They were maintained in tempera ture controlled room (21°C) with stable humidity (50-60%), fed ad libitum with commercially available rat pellet diet (EWOS Södertälje S eden) and had free access to tap water

The rats were randomly assigned to 4 groups of 10 rats (I-IV). After being weighed 3 of the groups (I-III) were castrated by simple orchiectomy sparing the epididymis and [...] fat pad (Kral & Tisell 1976) while one group (IV) was sham operated [u]sing the same scrotal incision and handling the testes to simulate the operative trauma. The operations were performed in pentobarbital anesthesia (Mebumal ACO Sweden). Because of pulmonary infec tion one rat was excluded from the sham operated group IV

The rats were allowed to recover for one week before [s]tarting the series of bi-weekly intramuscular injections of testosterone enantate (Testoviron-Depot Schering A G Berlin W Germany), or only the same volume of the vehicle sesam oil according to the following plan:

Castrated rat

Group I large dose: 2 mg of testosterone every 14 days
Group II small dose 0 [...] mg of testosterone every 14 days
Group III sham injection 0 1 ml of sesam oil every 14 days

Sham-operated rats

Group IV sham injection: 0 1 ml of sesam oil every 14 days

Table 1

Group	Mean weight in g (±S D)	Analysis of variance Groups	P
I Castrated—large doses of testosterone (n = 10)		I II	<0 001
Initial weight	96 9± 5 4	I–III	<0 001
Final weight	349.3±16 8	I IV	n.s
II Castrated—small doses of testosterone (n = 10)			
Initial weight	96.5± 6 5	II–III	<0 001
Final weight	301 1±16 4	II–IV	<0 001
III Castrated—sesam oil (n = 10)			
Initial weight	93 9± 4	III–IV	<0 001
Final weight	304 1±12 ?		
IV Sham op —sesam oil (n = 9)			
Initial weight	96 4± 2.		
Final weight	350 0±19.5		

The size of the larger dose calculated from recent data on testosterone secretion rates in rats (de Jong et al 1973 Free et al 1973) was chosen to simulate physiological levels of this hormone All rats were weighed once a week and the food reservoir three times a week to evaluate food intake After 12 weeks the animals were fasted over-night weighed and sacrificed by carotid ex sanguination in a well-defined pentobarbital narcosis (50 mg/kg) (Kral 1974) Fat depots from different anatomical regions were removed according to procedures previously described by Krotkiewski & Björntorp (1975) weighed and analysed for determination of fat cell size according to Sjöström et al (1971)

Muscle tissue Immediately after sacrifice before dissection of fat depots two specimens each of the white part of the gastrocnemius and the soleus muscle were dissected Access to the soleus muscle was gained through a sagittal incision from the most distal part of the Achilles tendon up to the level of the knee After division of the tendon the gastrocnemius was dissected free from the soleus thus uncovering the intermuscular fat pad which was removed Subsequently the soleus was prepared by trimming off excess fat and fascia before dividing it into two equal samples

The white portion of the gastrocnemius was localized at the level of the knee after incision of the skin Excess subcutaneous fat and fascia was trimmed off before excision of muscle tissue Macroscopically this muscle was homogeneously white in appearance in contrast to the dark red color of the soleus Microscopy was not performed at this stage

The samples were frozen directly in liquid nitrogen and stored at −80°C until analyzed for determination of enzyme activity and muscle fiber distribution One portion of the muscle specimen was cut in a cryostat at −20°C and stained for myofibrillar ATP-ase after preincubation at pH 10.3 for classification of type I (slow) and type II (fast) fibres For classification of the subgroups type IIA (fast twitch high oxidative FTH) and type IIB (fast twitch low oxidative FTL) both NADPH staining (Novikoff ... and ATP-ase staining with preincubation at pH 4.6 ... was used (Brooke & Kaiser 1970).

After homogenization and weighing in 20°C ... portion of the sample was further diluted in buffer ... 20 μl aliquots were added to 1 ml of test medium. ... enzyme activity determinations were performed ... fluorometric techniques using a Farrand fluorometer ? (Farrand Optical Co New York) The reac... catalyzed by the enzymes under investigation were coupled to NAD/NADP linked reactions, and the change in NADH or NADPH were monitored according to principles outlined by Lowry (197?).

The measurement were made under V_{max} conditions i e with an excess of substrate By running standard curves with known amounts of fluorescence the experimental readings could be quantified

The enzymes investigated in the present study ... Mg stimulated ATP-se (E C.3 6 1 4) (Thorstensson 1976) lactate dehydrogenase (E C 1 1 1 27) in one direction—(reduction of pyruvate to lactate) according to Sjödin (1976) and phosphorylase (E.C.2.4.1 1) according to Lowry (1957) as modified by Karlsson (1971)

STATISTICS

Conventional statistical method were utilized i e Student t-test and analysis of variance Significant differences between groups in analysis of variance were further examined according to Armitage (1971)

RESULTS

Effects of body weight and food intake (Table 1)

Compared to sham operated controls (Group IV) body weight was significantly lower in the castrated groups without substitution (Group III) The small dose of testosterone (Group II) did not seem to prevent this The large dose of testosterone (Group I) seemed on the other hand to be sufficient to maintain normal body weight compared to the sham operated control (Group IV)

Comparison of food intake revealed that the castrated rats without substitution (Group III) and with the smaller dose of testosterone (Group II) ate significantly less than the rats with the larger dose of testosterone (Group I) Moreover both groups II and III ate somewhat less than sham operated controls though the differences were not statistically significant (Table)

Fat cell weight (Table 3)

The castrated rats in group III had smaller fat cell weights in most of the investigated regions compared to the sham-operated ... The ...

...ence in cell weight was found in the caudal ...taneous adipose tissue depot. The rats treated ...he small dose of testosterone (Group II) did ...reveal any significant differences in fat cell ...t compared to sham-operated rats.

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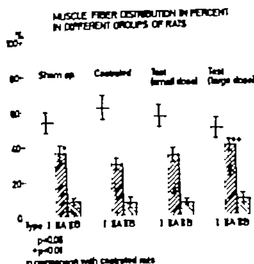


Fig. 1. The distribution (in percent) of slow twitch (type I) and fast twitch (type II A and type II B) muscle fibers in sham operated, castrated and testosterone treated (small and large dose) rats (9 rats in sham operated, otherwise 10 rats in each group). Significances calculated in comparison with castrated rats.

...USSION

...ation without substitution caused significantly ...ed weight gain in the prepubertal male rats in ...study in agreement with earlier studies ...stein 1941, Scow 1952, Kochakian et al. ...Kral & Traell 1976).

...e differences in body weight between the ...ps with and without substitution were not ap...nt during the first 5 weeks of study. It is inter...g to note that these differences occurred at the ...hen the differences in body weight between the sexes usually occur (at 7–8 weeks of age). Thus it is tempting to conclude that the differences in body weight between the sexes may be due to androgenic hormones. In view of the well known protein anabolic effects of testosterone even in intact sexually mature animals, the debut of the sex differences in growth and body weight at puberty may rather be interpreted as a result of both a stimulatory effects of testosterone in the male and an inhibitory effect of estrogens in the females. In contrast to male rats gonadectomized female rats gain more weight than intact animals (Kakolewski et al. 1968). Moreover, neonatal female rats when functionally oophorectomized by single injections of androgen, gain weight more rapidly than normal females (Swanson et al. 1963).

...le 2. *Mean food intake in castrated, sham op...ed and testosterone treated rat*

...rats of treatment	Food consumed g/day/rat (mean ± S.D.)
...up I. Castrated large dose (n=10)	19±3
...up II. Castrated small dose (n=10)	15±2
...up III. Castrated sesam oil (n=10)	15±2
...up IV. Sham operated—sesam oil (n=9)	18±
...lysis of variance ...d intake day/rat	
...-I	
...-II	
...-III	0.01
...-II	0.01
...-III	0.001
...-III	0.001

As judged by generally diminished fat cell weight castration seems to be associated with lower body fat content in comparison with normal or castrated and testosterone treated rat. Differences in the degree of fat cell weight decrease after castration changed the normal regional distribution pattern of adipocytes. This is explained by uneven emptying of fat cells. After castration the epididymal adipocytes decreased by 35% while subcutaneous adipocytes only by 9%. The lack of testosterone effect seems to be responsible for these changes as the

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Weeks of treatment	Food consumed g/day/rat (mean ± S D)
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Analysis of variance of intake day/rat	
I–I	
I–II	
I–III	0 01
–II	0 01
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–I m	0 001

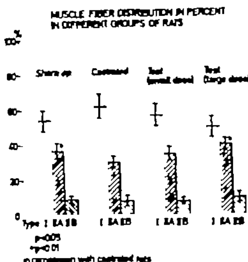


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Table 3

Group	Fat cell weight in different regions of adipose tissue mean ± S E			
	Perirenal	Inguinal	Epididymal	Caudal
I Castrated large dose of testosterone ($n=10$)	0.54±0 05	0.30±0 04	0 40±0 04	0 41±0 05
II Castrated small dose of testosterone ($n=10$)	0.50±0 06	0.31±0 04	0.35±0 04	0 37±0 04
III Castrated injected with sesam oil ($n=10$)	0.37±0 04	0 [illegible]±0 04	0 27±0 0	0.[illegible]5±0 0[illegible]
IV Sham operated ($n=9$)	0.55±0 06	0.25±0 04	0.37±0 04	0 4[illegible]±0 05
Analysis of variance	P	P	P	P
I II	n s	n s	n.s	n s
I–III	<0 01	n s	<0 01	<0 01
I–IV	n s	n s	n.s	n.s
II III	n s	n s	n s	<0 01
II–IV	n s	n s	n s	n.s
III–IV	<0 01	n s	<0 05	<0 01

large dose of testosterone restored the normal distribution pattern of fat cell weight

Similar selective decrease of fat cell size in epididymal depot after castration was recently found by Kral & Tisell (1976) As suggested author the localization of the epididymal [illegible] suggests a role in the reproductive function of testis and epididymis which could explain a [illegible] sensivity of the adipocytes in this depot [illegible] lack of testicular steroids"

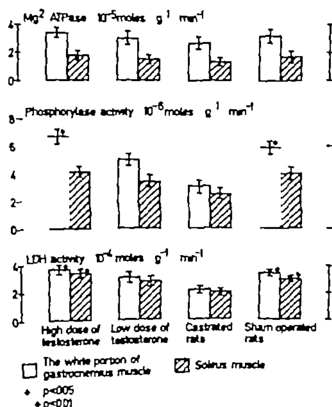


Fig 2 The activity of the Magnesium stimulated ATPase Phosphorylase and Lactate Dehydrogenase (LDH) in the white portion of gastrocnemius muscle and (predominantly red) soleus muscle The activities were estimated in sham operated castrated and testosterone treated (high and low dose) rats (9 rats in sham operated otherwise 10 rats in each group) Significances calculated in comparison with castrated rats

Castration caused a decrease in the proportion [illegible] FTH (type II A) fibres in white gastrocnemius [illegible] change in relative distribution pattern could be reversed by testosterone administration Tes[illegible]terone administration to female guinea pigs [illegible] been previously found to increase both muscle weight fiber size and relative proportion of [illegible] twich white muscle fibers (Gutman & Hanzlikova 1970) In this respect castration without substitution caused "a feminization" of the muscle fiber distribution pattern

The effect of testosterone on phosphorylase activity is in accordance with previously described increase of total muscle glycogen content after [illegible]tosterone treatment in rats (Adolfsson 1973) [illegible] cording to Newsholme (1978) the maximum rate [illegible] anaerobic glycolysis in any given muscle can [illegible] predicted from the maximum activity of phosphorylase (or phosphofructokinase)

Thus higher glycolytic activty higher gly[illegible] content and the postulated higher rate [illegible] glycogenolysis in testosterone substituted rats [illegible] be seen as in accordance with previously described testosterone effect on strength and muscle [illegible] (Ward 1973) As the reciprocal relationship of [illegible] phorylase and oxidative enzymes in skeletal muscle has been postulated previously (Dubowitz & Pearse 1960) the lack of bioche[illegible] [illegible]hanges in "red"

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Table 3

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Group	Perirenal	Inguinal	Epididymal	Caudal
I Castrated large dose of testosterone ($n=10$)	0.54±0.05	0.30±0.04	0.40±0.04	0.41 0.05
II Castrated small dose of testosterone ($n=10$)	0.50±0.06	0.31±0.04	0.35±0.04	0.37±0.04
III Castrated injected with sesam oil ($n=10$)	0.37±0.04	0.2_±0.04	0._7±0.0	0.25 0.0_
IV Sham operated ($n=9$)	0.55±0.06	0._5±0.04	0.37±0.04	0.4 ±0.05
Analysis of variance	*P*	*P*	*P*	*P*
I–II	n	n s	n s	n.s.
I–III	<0.01	n s	<0.01	<0.01
I–IV	n s	n s.	n s	n s
II–III	n s	n	n	<0.01
II–IV	n s.	n s.	n	n s.
III–IV	<0.01	n s	<0.05	<0.01

large dose of testosterone restored the normal di tribution pattern of fat cell weight

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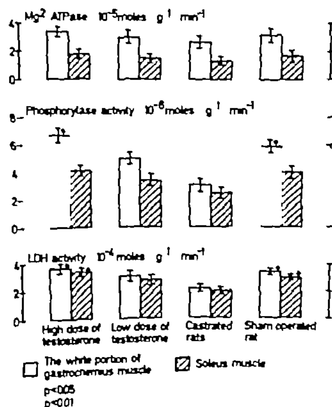


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Castration caused a decrease in the proport FTH (type II A) fibres in white gastrocnem change in relative distribution pattern could versed by testosterone administration Tes terone administration to female guinea pi been previously found to increase both musc weight fiber size and relative proportion of twich white muscle fibers (Gutman & Han 1970) In thi respect ca tration without subst caused a feminization of the muscle fiber tribution pattern

The effect of testosterone on phosphorylase tivity is in acc rdance with previously desc increase of total muscle gly ogen content after tosterone treatment in rats (Ad lf s n 197 cording to New holme (1976) the maximum r anaerobic glycoly is in any given muscle ca predicted from the maximum activity of ph phorylase (or pho pho fructokinase)

Thu higher glycolytic activity higher gl content and the postulated higher rat gly ogenoly i in test sterone substituted ra be seen as in a cordance with previously desc te t ter ne effect n strength and muscle (Ward 1973) A the reciprocal relation hip of ph rylase and oxidati e enzyme in skeletal ha been postulated previ usly (Dubowitz & P 1960) the lack of bioch anges in "red

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Key words: Kidney, carbonic anhydrase, urine acidification, bicarbonate reabsorbtion, diuretics, affinity chromatography, radioimmunoassay, stopped-flow technique

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The kinetic part of this work was reported at the Sixth International Congress of Pharmacology, July 20–25, 1975, in Helsinki.

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plasmic enzyme was isolated in highly purified form and shown to be probably identical with the high activity (with respect to CO_2) isoenzyme HCA–C found in the erythrocytes (Wistrand et al 1975) This enzyme here called CA–C has been isolated from cortex medulla and the papilla and appears to constitute the only soluble type of cytoplasmic carbonic anhydrase in the kidney (Wåhlstrand & Wistrand 1980) In the present paper the kinetic properties of this pure enzyme was studied at 37°C under near physiological conditions The access to a new radioimmunosorbent technique for assaying the levels of this enzyme in human tissues also offered a possibility to determine its levels in the various regions of the kidney The data obtained were used to estimate the quantitative role of the enzyme for the kidney function using an approach somewhat different from those used by the above cited authors but similar to the one used by Davies (1948) for the acid secreting cells of the stomach

MATERIALS AND METHODS

Preparation of kidney extracts

The kidneys used were fresh donor kidneys ready for transplantation There was no history of renal disease Thiazides or other drugs of importance had not been administered to the donors as far as it is known Immediately after removal the kidneys were perfused with 400–100 ml of cold Ringer-dextran type of solution (Perfadex® Pharmacia Uppsala, Sweden) containing 10 million IU benzylpenicillin 50 ml 2% lidocaine (Xylocain® Astra Sodertälje Sweden) and 5000 IU heparin per 1000 ml solution This was followed by perfusion with 400–1000 ml of 10% invertose solution (Inverdex Pharmacia, Uppsala Sweden) to which sodium bicarbonate had been added The perfusion pressure was 150 mm Hg The warm ischemic period varied between 30 and 60 min The kidneys were stored at 4°C and tissue preparation started within 4 h of perfusion Slices were taken from the cortical medullary and papillary region and gently homogenized in ice-cold 0 2 M sucrose An aliquot of the homogenate was taken for analysis of protein by the method of Lowry et al (1951) and of carbonic anhydrase isoenzymes (see below) The homogenate was centrifuged at 100000×g for 60 min. The particulate fraction and the fluffy top layer were removed The supernatant was dialyzed against 10 mM HEPES buffer pH 8 3 200 μM EDTA and frozen Its content of carbonic anhydrase isoenzymes and hemoglobin was then determined Hemoglobin was assayed by the cyan-methemoglobin method (Kampen & Zijlstra 1961). Due to the presence in kidney of other iron porphyrin substances this method probably overestimates the content of hemoglobin and therefore also the amount of contaminating blood in the kidney tissues by about 50% as found by Funakoshi & Deutsch (1971)

Preparation of purified renal soluble carbonic anhydrase

The cytoplasmic soluble enzyme from the pooled supernatants was purified by affinity chromatography as described previously (Wistrand et al 1975 Wåhlstrand & Wistrand 1980) The erythrocyte isoenzymes HCA-C and HCA–B were similarly prepared The adsorbant was made by coupling the inhibitor sulfanilamide to an epoxy-activated agarose via a long hydrophilic spacer prep according to the method of Sundberg & Porath (1974) The adsorbant is stable effective and selective for both low (HCA–B) and high (HCA–C) activity forms of human carbonic anhydrase isoenzymes The unspecific absorption is low and the enzymes eluted are highly purified The purified enzymes were stored in a refrigerator as a precipitate in saturated ammonium sulphate This prevents loss of significant enzyme activity during several months The enzyme concentration was calculated from the absorbance $A^{1\%}_{280}$ (cm) using a value of 18 7 Its molecular weight was taken to be 29800 (Wistrand et al 1975)

Kinetic methods

Substrate kinetics The kinetic properties of the purified renal soluble enzyme were studied at 37°C using the pH indicator optical stopped-flow technique The procedure as outlined by Khalifah (1971) was followed using indicator (p-nitrophenol indicator grade recrystallized four times in water) and buffer (N-methylimidazol E Merck A G Darmstadt GFR purified by distillation under reduced pressure) with similar pK values and allowing the reaction to occur during pH-changes of less than 0 06 pH units The measurements were made in a stopped-flow spectrophotometer (Durrum Instrument Corp Palo Alto Calif USA) equipped with a Teflon flow system 20 mm optical path length observation chamber and specially made tight drive-syringes suitable for work with gaseous substrates. The temperature was maintained by circulating water from a constant temperature bath around the drive syringes and the mixing and reaction chambers Substrate solution of CO_2 were freshly prepared by bubbling 100% CO_2 from a gas cylinder into distilled boiled water at the temperature of the stopped-flow apparatus It was diluted with CO_2-free deaerated water in a syringe to give the required substrate concentration The CO_2 concentrations were calculated from Henry's law by using the solubility coefficient of CO_2 as given by Harned & Davis (1943) Bicarbonate solutions were prepared freshly from crystals checked before use The CO_2 concentration in these solutions was determined using a CO_2-electrode (type 5036, Radiometer Copenhagen Denmark) The reaction was initiated by mixing equal volumes of the substrate with the buffer solution containing indicator and enzyme The ionic strength of the assay mixture was kept constant and physiological by adding sodium sulphate Sulphate was chosen because it has not been incriminated as catalyzing or inhibiting the reaction (Magid 1968 Khalifah 1971) Steady-state initial rates were obtained from the linear portions of the photographed reaction. Measurements were repeated usually three times with the same set of substrate buffer solutions. The initial rate used for calculation of the kinetic parameters was taken as an average of these determinations It was corrected for

the uncataly zed reaction Each set of the Michaelis ters as determined ith one enzyme-buffer solu- d between 6 and 12 different substrate solutions. ichaelis-Menten equation as fitted t the xperi- data by an iterative method with the aid of ear least-squares curve-fitting program, xecuted BM 370/155 digital computer The kinetic parame well as their standard deviations were then ob- It as found necessary to correct for the back due to significant amounts of CO_2 in the bicarbo- lutions This correction as made based on the data for the enzyme dehydration rate in the pres- CO_2 (De Vos & Kistiakowsky 1961)

nse kinetics Inhibition of the enzyme by sul- ide (Sigma C St. Louis Mo USA) thiazide (Merck, Sharp & Dohme Rahway N J acetazolamide (American Cyanamid C Pearl New York, USA) and sodium chloride as tudied inhibitor concentrations and 2–3 substrate concen- The data resulted in straight lines and indicated mpetitive type of inhibition (Fig. 2) when plotted ing to the graphic method of Dixon (1953) It was re felt justified to use the non-linear equation

v_i I)

ates the concentration of inhibitor which reduced zyme rate by half I_{50} and its standard deviation are the measured initial rates in the presence or e of total inhibitor concentration I respectively hibitor constant, K_i, was obtained from the relation $\frac{1}{2}$[E]$_t$ here [E]$_t$ is the total enzyme concentra-

t of arbonic anhydrase levels in he rious rts of the kidney

nic anhydrase proteins were analyzed in the renal nates or its supernatant by radio-immunosorbent ique (Wistrand et al. 1979) using antibodies selec- gainst the human erythrocyte isoenzymes HCA–C CA–C) and HCA–B (anti-HCA–B) The antibodies produced as previously described by Wistrand & 1969) Antibodies against the purified renal enzyme were also produced and found to react like anti- –C. This would be expected since HCA–C and the enzyme are antigenically and otherwise identical rand et al. 1975) The sensitivity of this method is 0 2 enzyme protein/ml of tissue fluid and the precision is n duplicate determinations. Recov ry experiments t he accuracy of the method have show for several of human tissues that the method detects all and 98% of HCA–C and HCA–B respectively (Wi- nd & Garg 1979)

c thonic anhydrase activity of the tissue homogenates s assayed by the changing pH-method of Philpot & pot (1936) This involves the determination of time to lower pH (10 to 7 4) of 1 ml of carbonate buffer as by change in color of phenol red at 0°C in 7 ml ne One enzyme unit is the amount of enzyme that ces the reaction time by half.

The molar concentration of the enzyme in the superna- was also titrated (Kernohan 1965) by use of the strong

Table 1 *Hydration and dehydration kinetic parameters for h man en l cytoplasmic carbonic anhydrase purified by affinity chromatography*

Conditions of test 25 mM N-methylimidazole buffer with added sodium sulphate to keep the ionic trength at 0 15 pH 7 1–0 03 0.01% peptone 50 μM p-nitrophenol 37°C±0 [E]$_t$ was 0 04 μM Six different inhibitor con- centrations w re tested again t 1 6 and 1 7 mM CO_2 or 5.0 and 20 mM HCO The inhibitor was present in both driv yringes and was mixed with enzyme in the buffer yringe about 2 min before the reaction started The parameters are given with their 95%-confidence limits within bracket k_u for the uncatalyzed hydration reac tion was 0 12 The dehydration second-order rat constant was 17 M 10 When multiplied by the ionization constant for H_2CO_3 $K_{H_2CO_3}$ taken to be 3 5 10 M we obtain alue for $k_{H_2CO_3}$ of 60

Kinetic parameter	Hydration	Dehydra- tion
K_m, mM	11 8 (6 9–16 7)	70
10^{-4} V/[E]$_t$	10 6 (8.1 13 1)	5
K_i, sulfanilamide μM	6 4 (5.0–7.8)	9 1 (6.5–11 7)
K_i, chlorothiazide μM	0 96 (0 72 1.20)	0.99 (0 85–1 13)
K acetazolamide μM	0 016 (0 005–0.028)	
K_i, sodium chloride mM	>200	–

(K = M) nhibitor etho zolamide (Upjohn Ltd Kalamazoo Mich USA). Each molecule of carbonic anhydrase binds one molecule of ethoxzolamide 1 these titrations an electrometric hanging pH-method (Wistrand & Kinne 1977) was used to assay the enzyme activity before and afte graded inhibition (details are given in the legend of Fig. 3).

RESULTS

Kinetics of the uncatal ed and at ly ed hydration and dehydration reactions t 37°C

Uncatalyzed reactions The rate constants for the hydration of CO_2 and the dehydration of H_2CO_3 at 37°C differ considerably in the literature as um marized by Garg and Maren (1972) Our values for k_{CO_2} and $k_{H_2CO_3}$ (Table 1) agree with those previous- ly reported for the stopped-flow technique where buffered media were used. Secondary phosphate has been claimed (cf Dennard & Williams 1966) to catalyze both the hydration and the dehydration reaction. Other investigators (cf Magid & Turbeck

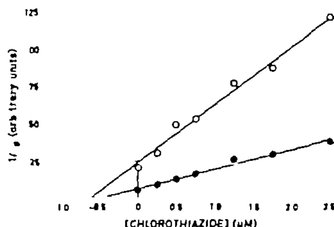


Fig 1 Inhibition of purified human renal cytoplasmic carbonic anhydrase by chlorothiazide Stopped-flow method Hydration reaction Enzyme $[E]_0 = 0.04$ μM Inhibitor was present in both drive syringes and mixed with the enzyme 30 min before assay The assay medium was 25 mM N-methylimidazole buffer pH 7 1 50 μM p-nitrophenol 0 01% peptone 37°C Ordinate reciprocal initial velocity arbitrary units abscissa concentration of chlorothiazide $[CO_2]$=1.56 mM (unfilled rings) or 7.34 mM (filled rings).

1968) found it to be without effect In our system the rate constants were not significantly different when measured in 25 mM phosphate or N-methylimidazole buffers at 37°C It is therefore not likely that phosphate has a physiologically significant effect on the uncatalyzed hydration of CO_2 or dehydration of H_2CO_3

Catalyzed reactions When data of Table 1 are compared with previous kinetic data collected at 25°C (Wistrand et al 1975) the only difference between the values of 25 and 37°C was the 1.5–? times larger turnover numbers at 37° The values for K_m however were not significantly changed by the temperature rise The data agree qualitatively with those of Kernohan (197?) who has made a systematic study on the effect of temperature on the kinetics of the purified bovine erythrocyte enzyme The data indicate that the observed increase in the enzymatic reaction rate is due to an increase in the turnover number whereas K_m is rather invariant with temperature

N methylimidazole has been reported by Khalifah (1971) to be non-inhibitory in the hydration reaction of HCA–C Strong phosphate buffers (>50 mM) have been reported to inhibit human erythrocyte enzymes (Kernohan 1965) in the hydration reaction although this was ascribed to impurities in the phosphate buffer used In contrast to these findings Christenson & Magid (1970) found that phosphate has a net activating effect on HCA–C in the hydration reaction at pH 7.3 In the present experiments a 25 mM phosphate buffer was not inhibitory or activating since we observed no significant difference between the K_m and turnover number of the enzyme when compared with the values collected in the N-methylimidazole buffers at 37°C It therefore appears unlikely that the phosphate concentrations in vivo will affect the catalytic activities of the renal enzyme

The kinetic parameters of the dehydration reaction (Table 1) agreed reasonably well with the calculated (DeVoe & Kistiakowsky 1961) from the hydration kinetic parameters and the Haldane relation The value for K_m agrees with that previously collected at 25°C in phosphate buffers by Gibbons and Edsall (1964) and by Wistrand et al (197?) The results were similar to those seen in the hydration reaction i e raising the temperature increased the turnover number without affecting the K_m

Inhibitor kinetics Sulfanilamide and chlorothiazide were tested in the hydration and the dehydration reactions giving curves which indicated the competitive type of inhibition (Fig 1). The inhibition by acetazolamide was non-competitive at hydration reaction Raising the temperature from to 37°C increased the K_i for sulfanilamide ? fold The effect of temperature on the inhibition by sulfonamides has not been studied systematically but seems to vary for different sulfonamides and perhaps also with the type of enzyme (Maren Wiley 1968) Chloride was not inhibitory (K_i>1 mM)

Levels of carbonic anhydrase in different regions of the kidney

The immunoassayable levels of CA–C and CA– the supernatants of kidney no 15 16 and 17 similar to those of the other kidneys where enzymes were instead titrated on the whole homogenate (see Table 2) In kidney no 6 and titrations were done on the supernatant as well on the homogenate The average ratio of the values for CA–C of the supernatant and homogenate 1 1±0 1 (S D n=4) These data therefore indicate that the enzymes in the homogenate remain in solution after centrifugation and were not adsorbed to the particulate fraction

The values for CA–C in cortex and medulla expressed as mg enzym[illegible] in per g tissue pr[illegible]

Immunoassayable levels of carbonic anhydrases B and C in various regions of the human kidney
... Hb as determined by the cyan-methemoglobin method of Kampen & Zijlstra (1961)

Levels in mg per g of extractable protein of homogenate

Cortex			Medulla			Papilla		
CA-B	CA-C	Hb	CA-B	CA-C	Hb	CA-B	CA-C	Hb
0.014	1.40			–			–	–
0.007	0.96		0.030	0.58		0.078	0.76	–
			0.330	1.78		0.100	0.21	
0.022	1.60							–
0.200	1.50		0.018	0.88	–	0.095	0.50	–
0.140	1.36					–		–
0.260	1.57		–					–
0.124	0.96	5.6	0.098	0.86	7.2	0.094	1.03	8.0
0.078	1.45	7.4	0.044	1.29	5.2	0.056	0.94	7.4
0.017	0.99	4.3	0.027	0.66	5.1	0.150	0.42	26.0
0.098	1.31		0.091	1.00		0.096	0.64	

...verage catalytic activities in cortex, medulla and papilla of these donor kidneys were 242, 194 and 157 enzyme ...wet tissue respectively as determined by the indicator changing pH-method of Philpot & Philpot (1936)

...ghtly lower than those 1.71 and 1.73 mg/g ...ed by Funakoshi & Deutsch (1971). This is ...ly explained by less contribution of HCA-C ...contaminating red cells in our perfused kid... The values for CA-C were highest in cortex ...ed by medulla and papilla in agreement with ...der of catalytic activities found in these re... (Table 2). The same order has been found for ...ual tubular cells, dissected out from the dif... parts of the single human nephron (Mat...mer et al. 1968).

...values correspond to 0.1 0.1 and 0.06 mg ...per g ...et tissue of cortex, med...lla and papil...pectively. Since the ...pecific activity of pure ...CA-C is 1520 enzyme units per mg (Wistrand ...1975) it may be calculated that cortex, medulla ...pilla should contain 182, 162 and 91 enzyme ...per g wet tissue respectively. These values ...well with those determined experimentally on ...regions (Table 2), using the same catalytic ...od for measuring the enzyme units. This indi... that the majority of antigenically active CA-C ...o catalytically active. This should rule out the ...bility that the kidney contains other high-ac... forms of the enzyme immunochemically dif... ...t from CA-C. Moreover the titration of the ...ber of catalytically active enzyme sites by the ...e strong inhibitor ethoxzolamide gave values ...h agreed rather well with those of the im...chemical method. The average ratio between ...ues of ethoxzolamid... nd antibody titrations

of 4 supernatants was 1.–±0.3 (S.D.). The results of an experiment on the inhibition of the enzyme activity of cortex and medulla of one kidney is seen in Fig. –. The activity of a fixed concentration of

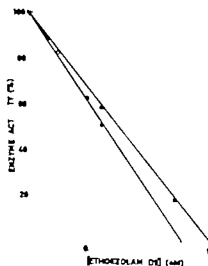


Fig. 2. Titration of renal carbonic anhydrase activity by the inhibitor ethoxzolamide. Electrometric method. Conditions of test. 10 mM phosphate buffer with 25 mM Na_2SO_4, 900 μM EDTA, 12.5 mg/ml bovine plasma albumin. $[CO_2]$ was 3.8 mM, initial pH 7.0, °C. 0.05 $[E]_0$ was 1.2 and 0.8 nM respectively as determined immunochemically on the supernatants of the cortical (open rings) and the medullary (filled rings) homogenate. Ordinate: activity as percentage of activity in absence of inhibitor. Abscissa: concentration of ethoxzolamide in nM

enzyme is plotted against the amount of inhibitor added The intercept on the inhibitor axis gives the value for the moles of enzyme present 1.3 nM in cortex and 1 6 nM in medulla In this experiment the values were slightly higher than those 0 8 and 1 ? nM respectively obtained by the immunoassay

We believe that all CA–B titrated immunochemically (Table 2) originates from contaminating blood From the hemoglobin values in the cortex medulla and papilla of kidneys no 15 16 and 17 (Table 2) it may be calculated that the kidneys contain less than 0 8% blood except for the papilla of kidney no 17 which contained ? 5% Since whole blood contains 12 1 μg HCA–B and 1.5 μg HCA–C per mg hemoglobin (Wåhlstrand et al 1979) it may further be calculated that all CA–B and less than 10% of CA–C originate from blood The levels of CA–C were rather similar in all kidneys and between 5–100 times higher than those of CA–B in all regions The levels of CA–B on the other hand varied strongly but were on an average the same in the different regions Funakoshi & Deutsch (1971) found no CA–B in cortex but 0 99 mg CA–B/g protein in the medulla of a non perfused autopsy kidney However these authors reported higher hemoglobin values than found here which makes the correction for blood contamination more difficult In a recent study (Wåhlstrand & Wistrand 1980) only CA–C could be isolated from the medullary and papillary regions of well perfused human donor kidneys

The presence of enzyme proteins in cortex medulla and papilla agrees with the finding that carbonic anhydrase activity is found histochemically along the whole human kidney nephron with exception for glomeruli and the straight part of the proximal tubule (Lönnerholm 1975)

DISCUSSION

The kinetic data on enzyme activity in highly diluted hemolysates have been shown to be applicable to conditions inside whole erythrocytes (cf Itada & Forster 1977) The enzyme activity of intact red cells is moreover inhibited by sulfonamides as can be predicted from inhibition kinetics of the purified isoenzymes in dilute solutions (Wistrand & Bååthe 1968) Thus there is evidence that both substrate and inhibitor kinetics are not changed when the enzyme is enclosed inside a cell membrane

Direct measurements of carbonic anhydrase activity and its inhibition in intact tubular cells have not been reported Estimates of the potential of carbonic anhydrase to catalyze the CO_2-reactions in these cells are more uncertain than for the erythrocytes due to the presence of carbonic anhydrase not only in the cytoplasm but also in the renal cell membranes Little is known about kinetic properties of this membrane-bound enzyme (cf Wistrand & Kinne 1977) However recent results from micropuncture studies have indicated that only the cytoplasmic enzyme is involved in the process of proximal tubular renal acidification (Karlmark et al 1979) This has encouraged us to try to estimate the quantitative role of the cytoplasmic enzyme in renal acidification using the data on its kinetic properties and cellular concentrations as collected in the present paper

Calculation of the capacity of the uncatalyzed and catalyzed CO_2 reaction to generate protons and neutralize alkali within the tubular cells

We assume that renal tubular cells actively secrete protons (Pitts 1958) leaving an alkaline cell interior to be buffered by the hydration of an abundant supply of CO_2 Direct support of this hypothesis is the finding that acid secreting separated cells (Struyvenberg et al 1968) and rabbit (Pallard et al 1979) renal tubules are relatively alkaline (… method) and become even more so about 0 1 unit after inhibition of carbonic anhydrase

The capacity of the uncatalyzed hydration of … within the tubular cells to generate protons was calculated from the equation

$$\frac{dCO_2}{d_t} = -k_{CO_2}[CO_2] + k_{H_2CO_3}\frac{[H^+][HCO_3^-]}{K_{H_2CO_3}}$$

where k_{CO_2} and $k_{H_2CO_3}$ are the rate constants … Table 1 and $K_{H_2CO_3}$ is the true ionization constant for carbonic acid taken to be $3.5 \cdot 10^{-4}$ M under in vivo conditions (cf Roughton 1964) Since the present kinetic data were collected at ionic strength and buffer capacity in the physiological range corrections (Jonsson et al 1976) for the small difference in buffer capacity between the buffer and tubular fluid were thought to be necessary … possible catalytic activity (base catalysis) of … cellular concentration of HPO_4^{2-} has been neglected as discussed in the Results section $[CO_2]$ was … to be … 0 mM (PCO_2 = 65 … G) and in diff…

...ium ...th the blood (Du Bose et al 1978) ...] as assumed to be 25 mM as measured ...y by Khuri (1979) in the rat late proximal ...ate tubule using an ion-exchange bicarbo-...nsitive microelectrode

...tituting these values into the equation at ...rium ...hen $dCO_2/dt = 0$ and solving for [H], ...n equilibrium ...alue for the intracellular pH ...5

...only available data on intracellular pH during ...ecretion are those just cited from the derived ...method. This pH must be on the alkaline side ...equilibrium value since only then is CO ...absorbed and hydrated inside the cell From ...tion of the equation it becomes obvious that ...te dCO_2/dt, of CO absorption—or proton ...ation—will depend on the assumed magnitude ...alkaline pH disequilibrium within the cell.

...e take the pH of the secreting cell to be 7.35 ...an alkaline pH disequilibrium (not to be con-...with the pH-disequilibrium of the luminal ...Rector 1973) of 0 10 pH unit application of ...uation shows that

$$... -0\ 1\ \sec \quad 0\ mM + 60\ \sec$$

$$\frac{25\ mM}{...30} = 50\ \mu M \times \sec \quad \text{or } 3\ mM \times min$$

...value becomes 0 13 mM × min If we instead ...me that the cell pH is 7.26, i.e. 0.01 pH unit ...e the equilibrium pH.

...e assume further that the uptake of CO_2 takes ...e in 200 ml of tissue fluid (2/3 of the weight of ...kidney) This is a generou estimate but takes ...account the possibility (Mello-Aires & Malnic ...) that the volume of proton generating tissue ...be larger than that of tubular cells. Under ...ition of good mixing and diffusivity of the ...tants it may then be calculated that the un-...lyzed reaction could generate 600 or ..5 μmoles ...rotons per minute dependent on whether we ...e an alkaline pH disequilibrium of 0 1 (pH 7.35) ...01 (pH 7.26) pH-units respectively

...he rate of the enzyme catalyzed reaction may be ...culated from the Michaelis-Menten equation, us-...the kinetic parameters of the enzyme (Table 1) ...its concentration (Table) in the tubular cell ...ter (0.7? of cell volume). The enzyme catalyzed ...e was calculated to be 6800 times higher than ...d of the uncatalyzed rate

The physiological rate of renal acidification

Unde... ...ormal condition In a helthy person of 70 kg the kidneys would have to generate 3 56 mol min of protons to be able to reabsorb all filtered bicarbonate and excrete the relatively small amounts of titratable acid (Bernstein 1958) Dependent on the alkalinity of the cell this is more than 6 (pH 7.35) or 120 (pH 7.26) times the calculated capacity of the uncatalyzed hydration of CO_2 to produce the number of protons necessary for the neutralization of the intracellular hydroxyl ions. However the need to accelerate the rate of the uncatalyzed hydration of CO_2 is easily met by the potential of carbonic anhydrase to catalyze this rate 6800-fold. This gives a large excess of enzyme in relation to physiological needs However as discussed above the magnitude of this excess is dependent on the alkaline pH-disequilibrium within the cell Direct measurements of the CO_2-equilibrium within the cell during acid secretion are therefore needed, before the quantitative role of the enzyme can be defined more strictly However the calculations do indicate that the activity of the cytoplasmic enzyme enables the tubular cell to sustain high rates of proton secretion sufficient for all demands of urinary acidifications while maintaining near CO_2-equilibrium and maximal buffering capacity within the cell This would appear to be the physiological role of the enzyme

Under carbonic anhydrase inhibition. Another approach to assessing the quantitative role of the enzyme is to see how graded enzyme inhibition relates to the physiological effects Bernstein (1958) found that dose of 10 mg/kg of acetazolamide given orally gives maximal 18 % reduction of the bicarbonate reabsorption and excretion of H +NH in humans (see model of Fig. 3) At the peak of this renal effect the concentration of unbound inhibitor in plasma, I_{free}, is about 10 μM (Lehmann et al 1969). In the renal cortex it is probably 3 times higher due to accumulation during active renal secretion of acetazolamide (Maren 1968) If we therefore take I_{free} in the tubular cell to be 20 μM the fractional enzyme inhibition will be 0 9993 as calculated from the relation

$$i = \frac{I_{free}}{I_{free} + K}$$

This degree of inhibition would reduce the potential of the enzyme t accelerate the hydration of

enzyme is plotted against the amount of inhibitor added The intercept on the inhibitor axis gives the value for the moles of enzyme present 1 3 nM in cortex and 1 6 nM in medulla In this experiment the values were slightly higher than those 0 8 and 1 2 nM respectively obtained by the immunoassay

We believe that all CA–B titrated immunochemically (Table 2) originates from contaminating blood From the hemoglobin values in the cortex medulla and papilla of kidneys no 15 16 and 17 (Table 2) it may be calculated that the kidneys contain less than 0 8% blood except for the papilla of kidney no 17 which contained 2 5% Since whole blood contains 12 1 μg HCA–B and 1.5 μg HCA–C per mg hemoglobin (Wåhlstrand et al 1979) it may further be calculated that all CA–B and less than 10% of CA–C originate from blood The levels of CA–C were rather similar in all kidneys and between 5–100 times higher than those of CA–B in all regions The levels of CA–B on the other hand varied strongly but were on an average the same in the different regions Funakoshi & Deutsch (1971) found no CA–B in cortex but 0 99 mg CA–B/g protein in the medulla of a non perfused autopsy kidney However these authors reported higher hemoglobin values than found here which makes the correction for blood contamination more difficult In a recent study (Wåhlstrand & Wistrand 1980) only CA–C could be isolated from the medullary and papillary regions of well perfused human donor kidneys

The presence of enzyme proteins in cortex medulla and papilla agrees with the finding that carbonic anhydrase activity is found histochemically along the whole human kidney nephron with exception for glomeruli and the straight part of the proximal tubule (Lönnerholm 1975)

DISCUSSION

The kinetic data on enzyme activity in highly diluted hemolysates have been shown to be applicable to conditions inside whole erythrocytes (cf Itada & Forster 1977) The enzyme activity of intact red cells is moreover inhibited by sulfonamides as can be predicted from inhibition kinetics of the purified isoenzymes in dilute solutions (Wistrand & Bååthe 1968) Thus there is evidence that both substrate and inhibitor kinetics are not changed when the enzyme is enclosed inside a cell membrane

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Calculation of the capacity of the uncatalyzed catalyzed CO_2-reaction to generate protons and neutralize alkali within the tubular cells

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The capacity of the uncatalyzed hydration of CO_2 within the tubular cells to generate protons [illegible] calculated from the equation

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where k_{CO_2} and k_{H,CO_3} are the rate constants [illegible] Table 1 and K_{H,CO_3} is the true ionization constant for carbonic acid taken to be 3 5 10 [illegible] M under in vivo conditions (cf Roughton 1964) Since the present kinetic data were collected at ionic strength and buffer capacity in the physiological range [illegible] corrections (Jonsson et al 1976) for the small difference in buffer capacity between the buffer [illegible] tubular fluid were thought to be necessary [illegible] possible catalytic activity (base catalysis) of the cellular concentration of HPO_4 has been neglected as discussed in the Results section $[CO_2]$ was taken to be 0 mM (PCO =65 mm HG) and in [illegible]

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$$-0.12 \text{ sec} \quad 2.0 \text{ mM}+60 \text{ sec}$$

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Under normal conditions In a helthy person of 70 kg the kidneys would have to generate 3.56 mol min of protons to be able to reabsorb all filtered bicarbonate and excrete the relatively small amounts of titratable acid (Bernstein 1958). Dependent on the alkalinity of the cell this is more than 6 (pH 7.35) or 170 (pH 7.26) times the calculated capacity of the uncatalyzed hydration of CO to produce the number of protons necessary for the neutralization of the intracellular hydroxyl ions. However the need to accelerate the rate of the uncatalyzed hydration of CO_2 is easily met by the potential of carbonic anhydrase to catalyze this rate 6800-fold. This gives a large excess of enzyme in relation to physiological needs. However as discussed above the magnitude of this excess is dependent on the alkaline pH-disequilibrium within the cell. Direct measurements of the CO_2-equilibrium within the cell during acid secretion are therefore needed before the quantitative role of the enzyme can be defined more strictly. However the calculations do indicate that the activity of the cytoplasmic enzyme enables the tubular cell to sustain high rates of proton secretion sufficient for all demand of urinary acidifications while maintaining near CO_2-equilibrium and maximal buffering capacity within the cell. This would appear to be the physiological role of the enzyme

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$$= \frac{I_{free}}{I_{free}+K_i}$$

This degree of inhibition would reduce the potential of the enzyme to accelerate the hydration of

TUBULAR CELL WATER

[E] 8 μM — [I free] 20 μM

2.0 mM H_2O CO_2 ⇌ (7.2 min⁻¹ / 3600 min⁻¹) 3.2 μM H_2CO_3 ⇌ 25 mM HCO_3 pH 7.35 H^+

MEMBRANE

2910 μmoles min UPTAKE — 2932 μmoles min⁻¹ SECRETED

CO_2 ← H_2CO_3 ← H Y — HCO_3^- ← 2900 μmoles min⁻¹ FILTERED

EXCRETED — 22 μmoles min — 600 μmoles min

URINE

Fig 3 Model of proton generation and bicarbonate reabsorption in the human renal tubule after an apparent maximally effective dose of acetazolamide Values on rates of excreted bicarbonate and titratable acid are at ~3 h after 10 mg/kg of acetazolamide given orally to healthy humans (Bernstein 1958) Values on [CO_2] (Du Bose et al 1978) and [HCO] (Khuri 1979) are from direct measurement on rat kidneys pH was assumed to be 7 35 i e 0 1 pH unit on the alkaline side of the equilibrium value of 7.25 (see text) [H CO] is the calculated value at pH 7.35 Inhibition of carbonic anhydrase raises the cellular pH by about 0 1 pH unit (Struyvenberg et al 1968) and thereby increases the rate of the uncatalyzed hydration of CO_2 to 600 μmol min (see text) This rate can only support 17% of the absorption of all filtered bicarbonate However carbonic anhydrase can accelerate the rate 6 800-fold Therfore even if 99 93% of the enzyme is inhibited at an I_{free} of 20 μM (see text) there is still a 5-fold magnification (6 800×0 0007) of the uncatalyzed rate which would be enough for the reabsorbtion of the bicarbonate 910 μmol min not excreted in the urine after this dose of acetazolamide The protons generated intracellularly may be directly secreted (Pitts & Alexander 1945) or used to buffer the alkaline cell interior created by the cell acid secretion as discussed in the text

CO_2 from 6 800-fold to 5-fold (6 800 0 0007) However during carbonic anhydrase inhibition the cellular alkaline pH-disequilibrium increases by about 0 1 pH unit (Struyvenberg et al 1968 Paillard et al 1979) The uncatalyzed hydration of CO will then generate at least 600 μmol min of protons A 5-fold magnification of this rate would thus still be sufficient to support the absorption of 910 mol min of bicarbonate which continues to take place even after this apparently maximal dose of acetazolamide (Fig 3) It would therefore appear as if the increased cellular alkalinity makes the cell escape the effects of enzyme inhibition by increasing the rate of the uncatalyzed reaction by about 20-fold However when 100 μM of acetazolamide was added to the luminal perfusate in micropuncture studies of rat tubules in vivo (Lucci et al 1979) or in separated rabbit tubules (McKinney & Burg 1977) the proximal bicarbonate reabsorption became reduced by 85–100% If acetazolamide diffuses freely into the tubular cell from the luminal side this high concentration would inhibit the enzyme by 99 98% if the rat renal enzyme is kinetically similar to the human enzyme (Wistrand & Wåhlstrand 1977) [illegible] this case no enzyme activity should be left in the tubular cell (acceleration less than 2-fold) and the small amounts (<15%) of bicarbonate reabsorbed under these conditions probably reflect the [illegible] of the uncatalyzed hydration of CO These [illegible] tion data support the notion that the reabsorption of all filtered bicarbonate and the excretion of titratable acid is dependent on the hydration of CO_2 and its catalysis by cytoplasmic carbonic anhydrase Maren (1968) however found that despite they were given increasing doses of inhibitors with highly different structures did not [illegible] more than 20–35% of the filtered bicarbonate [illegible] was taken as evidence that most (65–80%) of filtered bicarbonate is reabsorbed by a separate mechanism On the other hand Mathiesen et al (1979) found a linear re[illegible]ip between bicar[illegible]

absorption and log intra enous doses of mg/kg body wt of acetazolamide in dogs estion whether all bicarbonate reabsorp- dependent on carbonic anhydrase activ herefore still open and will probably not lved until direct measurements of enzyme in the tubular cells have been done after ration of inhibitors. The use of irreversible rs for this purpose should be advantageous

o thank Staffan Lindahl for performing the kinetic ments, Karl-Gösta Kneuttila for performing the chemical titrations, and the colleagues at the Department of the University Hospital Uppsala, ding the kidneys. The work as supported by the Medical Research Council grant no. 2874

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..ation of pial arterial vessels in hypercapnia ..in acute hypertension

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AUER, L. M. & JOHANSSON, B. B. Dilatation of pial arterial vessels in hypercapnia and in acute hypertension. Acta Physiol Scand 1980. 109: 49–251. Received 30 Oct. 1979. ISSN 0001-6772. Department of Neurosurgery, University of Graz, Austria and Department of Neurology, Sahlgren Hospital, Göteborg, Sweden.

Pial arterial vessels were inspected in hypercapnia ($PaCO_2$ 97±9 (S.E.) mmHg) and in acute hypertension induced by 5 $\mu g\ kg^{-1}$ noradrenaline i. (mean arterial pressure 210±5 mmHg) by means of cranial window. The diameter of arterial vessels with resting diameter of 10 to 150 μm was measured either from photographs or by aid of an image splitting eyepiece. Arterioles with resting diameter ≤30 μm exhibited the highest degree of dilatation both in hypercapnia and acute hypertension. The mean dilatation was higher in hypertension than in hypercapnia in vessels of all sizes, the difference being statistically significant in arterioles up to 50 μm resting diameter.

Key words: Acute hypertension, hypercapnia, pial artery diameter, cranial window

..arbon dioxide dilates pial arteries is an old ..ation (Donders 1849). A number of studies ..shown that cerebral blood flow (CBF) in..s several hundred percent during hypercap..animals and man (for references see Auer ..) An abrupt increase in blood pressure of a ..tude that surpasses the capacity of the cere..essels to autoregulate also increases CBF but, ..a concomitant diffuse vasodilatation is in..not up to such high values as in hypercapnia ..sson & Nilsson 1979). This is in agreement ..he observation that CBF is heterogeneous in hypertension (Johansson 1974, Bill & Linder ..Intravital inspection of pial arteries during hypertension or drug-induced acute hyperten.. has revealed condition of alternating vaso..tion and relative vasoconstriction, so-called ..ge-string arteries (Byrom 1954, Rodda & Den..rown 1966, MacKenzie et al. 1976, Auer 1977). ..dilated parts increase in number when the ..ure is kept high for a longer period of time ..ating that more and more smooth muscle cells ..to the high intraluminal pressure with time. ..Dilatation of cerebral vessels in acute hyperten.. which usually leads to a transitory dysfunction ..e blood-brain barrier has been called "vaso-distension" overstretching or forced dilatation thus assuming a dilatation more pronounced than that produced by complete relaxation of the smooth muscle cells (Häggendal & Johansson 1972, Johansson, Strandgaard & Lassen 1974). However, the degree of pial vasodilatation present during acute hypertension and hypercapnia respectively has not been compared.

In the present investigation the responses of pial arterial vessels to hypercapnia and to acute hypertension were compared in order to answer the following questions. ( ) Is it vessels of the same size that react most to both stimuli? (*b*) Which dilatation is strongest?

MATERIAL AND METHODS

Ten cats with body weight of 1.5–3.0 kg were anesthetized with 30 mg kg sodium pentobarbital, intubated endotracheally and ventilated with 3:1 mixture of N_2O: O_2 with Loosco respirator. The left femoral artery and vein were cannulated for continuous monitoring of blood pressure (Statham transducer P23dB, Hellige Electromanometer, Rikadenki penwriter) and administration of drugs. A cranial window was made in the right parietal region and pial arterial vessels were investigated by measuring diameters from photographs or by aid of an image splitting eyepiece. (For details, see Auer 1978a.)

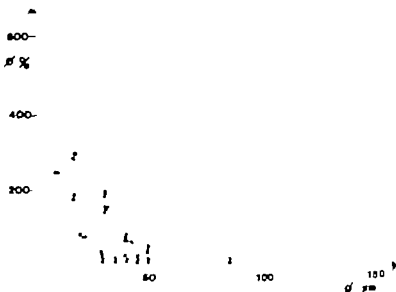


Fig 1 The percentual vasodilatation in acute hypertension (O) and in hypercapnia (■) plotted against the resting diameter in pial arterial vessels up to 150 μm The strongest reaction is seen in small vessels both in hypertension and hypercapnia.

Hypercapnia was induced in 5 cats by adding 10–15% CO_2 to the respired gas mixture thus increasing $PaCO_2$ from 30–41 mmHg (mean 37 8 mmHg) to 73–1 4 mmHg (mean 96 6 mmHg) The maximum dilatation was measured on arteries and arterioles with a resting diameter of 10 to 150 μm

Acute arterial hypertension was induced in 5 other cats by intravenous injection of 5 μg kg noradrenaline The mean arterial pressure (MAP) rose within 10–20 s from a mean of 98 mmHg to 210±5 mmHg (1‸2% increase) returning to normal within about 10 min

The extent of arteriolar dilatation during hypercapnia and hypertension respectively was compared in 3 groups of arterial essels with a resting diameter of 1–30 μm 31–50 μm and 51–150 μm. Differences in the degree of dilatation were evaluated with the Student s t test

RESULTS

During acute hypertension arteries and arterioles dilated mostly sausage string-like and less frequently in a diffuse manner whereas during hypercapnia dilatation was regularly diffuse Both in hypercapnia and hypertension a reciprocal relation between resting vessel size and degree of dilatation was observed similar to an exponential curve (see also Auer 1978*a b*) Thus the smallest vessels exhibited the highest and the largest the lowest degree of dilatation The percentual vasodilatation plotted against the resting diameter is shown in Fig. 1 It is evident that during acute hypertension (circles) the vessels—especially the dilated portions of "sausage string" vessels—had a larger diameter than during hypercapnia (squares) some instances an increase in number or l of the dilated parts was seen as a function of d tion of hypertension The mean dilatation larger in hypertension than in hypercapnia sels of all sizes with statistically significant ference in arterioles up to 50 μm resting dia (see Table 1)

DISCUSSION

Our results thus show that it is the smaller arterial vessels that have the largest chang diameter both in hypertension and hyperc The observation that dilatation during hyperte exceeds that in hypercapnia is in agreemen

Table 1 *The percentual dilatation of arteri sels of various esting diameters after acute tension and hypercapnia*

Mean values ± S E *n* = number of measurements

	Diameter (μm)		
	0–30	31 50	[illegible]
Hypercapnia	137±79 (*n*=70)	45±6 (=15)	[illegible]
Hypertension	239±19* (*n*=46)	1 1±16 (*n*=22)	[illegible]

Significantly different from hypercapnia

cept of overstretching and forced dilatation
rial vessels in acute hypertension. The
t pial arterial vessels are those that react
rongly with both dilatation and constriction
abrupt increase in blood pressure (Auer
Interestingly it is the same size of meninge
cerebral arterial vessels that shows the most
nced increase in the ratio between media
ss and radius in chronic hypertension in the
rdborg & Johansson 1979). The strong reac
a pial arterioles with a diameter less than
suggests that these vessels are of importance
cerebrovascular resistance something that
en the subject of considerable debate (for
ion see Purves 1978).

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hy intermittent ischemia in renal cortex ıg tourniquet shock in dog

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TYSSEBOTN I and KIRKEBØ A Patchy intermittent ischemia in renal cortex during tourniquet shock in dog Acta Physiol Scand 1980 109 253–260 Received 6 Nov 1979 ISSN 0001-6722. Institute of Physiology University of Bergen, Norway

The effect of bilateral hindlimb occlusion on the distribution of renal cortical blood flow was investigated in anesthetized dogs. Local blood flow in outer cortex (OCF) and in inner cortex (ICF) was measured by 6 platinum electrodes recording hydrogen gas clearance Renal blood flow fell insignificantly while hematocrit (Hct) rose from 37 to 47% during the 4 h tourniquet period. After the release of the tourniquets average arterial blood pressure fell to about 75% of control, whereas OCF and ICF were proportionately reduced towards 30–40% of control Hct increased to 58% Patchy intermittent ischemia in outer and inner corte was irregularly observed after release of the tourniquets when Hct had increased markedly as previously found during dehydration and endotoxin shock. The sudden shifts in washout rates from single electrodes were not accompanied by corresponding variations in total renal blood flow Heparinization or α-receptor blocking did not suppress the appearance of abrupt flow changes in this period. However sudden shifts in local flow were also seen shortly after application of the tourniquets, before Hct had increased, in this phase possibly induced by an increased renal nervous tone. In conclusion: OCF and ICF fell to the same degree during tourniquet shock. Patchy intermittent ischemia was observed in the renal cortex, most frequently at high Hct, but also before Hct did rise

Key ords Renal blood flow distribution hydrogen clearance tourniquet shock, blood flow heterogeneity

e of the frequent kidney impairment following atic shock (Rosenberg et al. 1971), little in- tion is available on the renal blood flow and tribution in such states. During control condi- and hemorrhagic hypotension, with a low locrit (Hct) we have previously found a geneous cortical blood flow (Aukland et al However during dehydration and endotoxin t, cortical blood flow changed to a pattern of ly intermittent flow variations when arterial l pressure (AP) and renal blood flow were re d and Hct was raised above 50–55% (Kirkebø yssebotn 1977 1980). This phenomenon was rved irregularly and often disappeared at very AP and renal blood flow These two models fore, ere not suitable for investigating possi- echanisms that could explain the sudden shifts ood flow

It is known that release of tourniquets after bilat eral hindlimb occlusion causes a state of circulatory shock (Stoner 1961) in which AP and renal blood flow are reduced concomitantly with a rise in Hct (Takács et al 1962) Trueta et al (1947) also have described patchy ischemic necrosis of outer renal cortex and postulated a redistribution of renal blood flow during tourniquet shock. We therefore decided to study the local and total renal blood flow during and after total occlusion of both hindlimbs in dogs, particularly to see if the previously described patchy intermittent flow is a general consequence of raised Hct and reduced AP and renal blood flow during shock.

METHODS

The effect of bilateral hindlimb tourniquets on distribution of renal cortical blood flow was studied in 15 dogs of both

Table 1 *Mean arterial pressure (AP) hematocrit (Hct) total (RAF) and local (OCF ICF) renal blood [illegible] in control at the end of the hindlimb occlusion period and after release of the tourniquets*
In Series B the tourniquets were applied before start of surgery Mean ± S D

	AP (mmHg)	RAF (ml/min g)	OCF (ml/min g)	ICF (ml/min g)	Hct (%)
Control $n=7$	1[illegible]5±[illegible]2	3 75±0 28	3 63±0 61	3 03±0 77	37 [illegible]
End of tourniquet period					
Series A $n=7$	124±17	3.24±0 61	3 06±0 77	2 82±0 77	4[illegible] 7
Series B $n=8$	130±16	3.52±0 65	[illegible]98±0 91	[illegible]59±0 60	[illegible] 7
Series A+B $n=15$	1?7±16	3 38±0 69	3 0[illegible]±0 82	2.70±0.67	[illegible]
Tourniquet off					
1/4 h $n=15$	103±17*	[illegible]28±0 86	1 91±0.58*	1 82±0 51	
1 h $n=14$	91±18*	1 75±1 16	1.56±0 ?9*	1.54±0.38*	[illegible]
2 h $n=14$	9?±[illegible]2	1 63±1.33	1 17±0 47*	1.23±0.57*	

* Significantly lower than control $P<0\ 05$

sexes weighing 15–30 kg. The dogs had free access to drinking water but food was restricted 18 h before surgery Anesthesia was induced by i v Nembutal [illegible]5 mg/kg b wt and additional doses given when needed The dogs were intubated with a cuffed endotracheal tube and breathed spontaneously

The kidney was exposed retroperitoneally through a flank incision and gently dissected free The renal artery was cannulated by a polyvinyl catheter with the tip directed upstream for injections of hydrogen saturated saline. Polyethylen catheters were put into the brachial vein and artery for infusions and AP recording respectively The AP was measured with a Hewlett Packard transducer and recorded on a Hewlett Packard recorder model 7796A Total renal blood flow (RAF) was measured by an electromagnetic flowprobe (Nycotron) on the renal artery and the flowmeter calibrated on a femoral artery

L shaped platinum electrodes with 0.5 mm diameter (sensitive cone tip diameter 0.5 to 0 1 mm and 1 mm length) were inserted in the renal cortex so that 3 electrodes had the sensitive tip in the outer half and 3 had the tip in the inner half of cortex The electrode shafts were sutured to the renal capsule The electrodes were polarized with + 0.[illegible] V against an Ag/AgCl referance electrode placed subcutaneously on the hip Hydrogen saturated saline with a temperature of 37°C was injected in the renal artery until a stable current was obtained on all electrodes Then the injection was abruptly stopped and the washout curve recorded on a 6 channel Rikadenki recorder (A kland et al 1973) The monoexponential washout curves were plotted on a semilogarithmic paper and the flow rates were calculated from the formula $F=-\lambda \ln 0.5/T\frac{1}{2}$ ml/min g where $T\frac{1}{2}$ is the half life of hydrogen concentration in tissue and the tissue/blood partition coefficient λ is 1 0

Experimental procedure

In 7 of the animals the surgery and the measurements of control blood flow were completed before the tourniquet were applied Series A

In 8 experiments the tourniquets were applied before the surgery started Series B

The wire tourniquets were placed on both hind [illegible] near the hip as possible and strongly tightened [illegible] blood flow The occlusion lasted for 4 h. The RAF and [illegible] were recorded continuously while measurements [illegible] local cortical flow were repeated each 20–30 min [illegible] out the tourniquet period. The two tourniquets were [illegible] relased with 5–15 min interval and local cortical [illegible] measurements repeated each 5–15 min for at least [illegible] tourniquet release (except in dogs). Since [illegible] saline infusion in the renal artery would tend [illegible] Hct hydrogen gas was administered [illegible] tracheal tube at RAF below 50% of control values [illegible] parison of locally injected and inhaled H_2-gas [illegible] showing no discrepancies at flow rates of 2 [illegible] less.

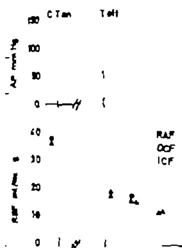
Hct was measured each hour and the total [illegible] proteins measured by the amido black method [illegible] Plum et al 1955) in the control period during [illegible] and several times after tourniquet release

In 5 dogs showing patchy intermittent cortical [illegible] during development of shock phentolamine [illegible] b wt) or phenoxybenzamine (ca. 3 mg/kg b. [illegible] ven in the renal artery to test whether the changes [illegible] persisted or disappeared. Similarly heparin [illegible] was given in 5 dogs.

At the end of each experiment the kidney was [illegible] drained for 2 min and weighed The drained kidney [illegible] was used for calculations of RAF in ml/min g The [illegible] tion of each electrode tip was carefully examined [illegible] cal evaluations were performed with Student's t [illegible]

RESULTS

In 7 experiments (Series A) control renal blood [illegible] was measured about 15 min prior to application [illegible] the tourniquet The results are given in Table [illegible] Local blood flow in outer cortex (OCF) and [illegible] cortex (ICF) were 3 63±0 61 and 3 03±0.7[illegible] min g respectively as [illegible] ted from 2 [illegible]



Effect of hindlimb occlusion on mean arterial pres-
(P) and renal blood flow (RBF) in control period (C)
placed tourniquet (T on to T off) and after release
...ments.

...ashout curves obtained with about 5 min in-
... in each dog. Hct averaged 37±2% and total
... in plasma ...veraged 5.24±0.95 g/100 ml
... In the control period all cortical washout
... ere monoexponential down to at least 10%
...tial saturation and abrupt changes in flow
... were never seen before tourniquet applica-

...urement during hindlimb occlusion

... the wires around the hindlimb were tight
... the dogs started to hyperventilate and the AP
...ased although adequate anesthesia was main-
...d as estimated by abolished eye lid and limb
...rawal reflexes. Within 30 min the AP in-
...ed 17% (to 146 mmHg) as an average for the 7
... and the renal arterial blood flow (RAF) de-
...ed 15% indicating that the renal vascular re-
...nce increased about 35% most likely mediated
... sympathetic nervous system. Ventilation and
... were normalized in about one hour but RAF
... returned to control level during the 4 h tour
...et period.

...than 30 min before release of the tourniquet the
... values were slightly reduced as shown in
...le 1. The average AP was unchanged from
...rol, whereas Hct had increased by nearly 30%.
... 8 other experiments (Series B) the tourniquets
... applied before starting surgery and the first
...urements in this series were performed the last half hour before tourniquet release showing blood flow and AP nearly identical with the values given for Series A. Because of this and since the experimental procedures after tourniquet release were also identical for the two series the following results for both groups are described in common as shown in Fig. 1 and Table 1.

During the tourniquet period local cortical blood flow measured with H_2 clearance sometimes showed striking irregular patterns. The washout curves from single electrode sampling sites incidentally showed abrupt changes to a lower or a higher clearance rate. Fig. 2 shows H_2 washout curves recorded simultaneously from 4 different sampling zones in different cortical depths in one kidney. The sampling site represented by curve A showed normal saturation of hydrogen but during washout sudden shifts in rate were observed (marked with arrows). Each of these different rates were linear during replotting and allowed calculation of flow. The curve B representing another area in cortex, showed a steady slow increase in zero-line before H_2 administration, and for some time during the H_2 injection. Then suddenly the area started to take up H_2 at normal rate (marked with arrow) and later showed a monoexponential washout curve. The interpretation must be that the area started with a blood flow close to zero and that the blood flow abruptly increased and remained high during the washout period.

During the tourniquet period such abrupt changes in the H_2-washout curves were observed in all except 3 animals, but in 7 dogs only on one electrode. The total frequency of abrupt changes was slowly increasing in this period, but sudden shifts in flow were seen also in 4 dogs shortly after the application of the tourniquet, before Hct had increased. Though sudden local flow changes could be observed on several electrode sites, concomitant sudden changes in RAF were not detected.

A rise in Hct by 25% indicates an appreciable loss of fluid from plasma. Edema was observed on the occluded limbs in only two dogs in the tourniquet period. During the whole tourniquet period the hindlimb temperature decreased in all dogs approaching the room temperature of 22°C.

Measurements shortly after release of the tourniquets

When the tourniquets were released from both hindlimbs after 4 h of occlusion all dogs started

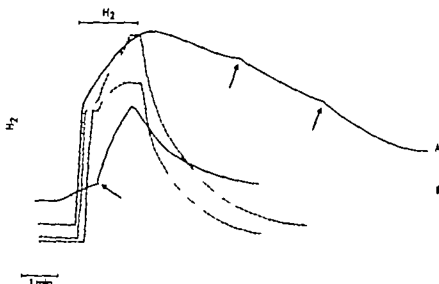


Fig 2 H washout curves of 4 single electrodes in one dog where the hindlimbs were occluded 10 min previous to the H administration Two of the curves show sudden bends (arrows) See text

to hyperventilate and the AP began to fall One dog died from heart arrest 25 min after release of the slings In the period between release and heart arrest in this particular dog the local cortical blood flow was patchy and RAF and AP steadily decreasing Hct increased to 68% within 20 min after tourniquet release indicating rapid fluid loss from circulated blood vessels

All other dogs (14) lived for at least 3 hours and the experiments were continued up to 7 hours after tourniquet release In all dogs pronounced edema was seen in the hindlimbs

Measurements during the first half hour a tourniquet was released showed an abrupt fa RAF OCF and ICF to 61 53 and 60% of con while AP fell to 82% all significantly lower control (Table I Fig 1) Renal vascular resi was calculated to be about 35% higher than co but about the same as shortly after tourniquet a cation

Local blood flow heterogeneity

In 7 dogs abrupt changes in washout rate s shortly after release of tourniquets as in Fi

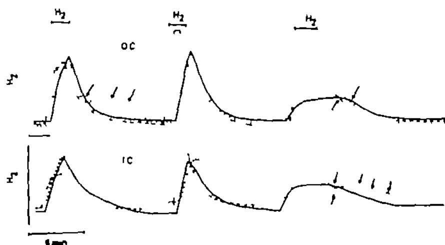


Fig 3A H_2 washout curves of 3 single electrodes in outer (OC) and 3 in inner (IC) cortex of a dog The first H administration was made about 5 min after tourniquet release The curves show independent sudden changes in washout rate (arrows) both in IC and OC (see text)



Fig. 3 B. H_2 washout curves of electrodes in outer (OC) and inner (IC) cortex. The H saturation started about 32 min after tourniquet release. When saline was infused the washout started at different time on different electrodes (arrows) (see text).

three consecutive washout curves for 6 elec in one dog are shown. The washout curves recorded 5 min after release of the slings of the curves showed constant flow while otted curve in the upper panel described 4 ent flow rates. This sampling area started out at a rate of 2.18 min fell abruptly to at the first arrow then started a slow wash- next arrow) (ca. 0.30 min) before it again at ird arrow increased the washout rate to 1.30 Each single component of the washout curve monoexponential.

e next series of washout curves showed well rved blood flow and fairly homogeneous blood in all sampling areas whereas the third wash- urve showed an inhomogeneous flow pattern ower flow in all tested areas and as well a lower The solid curve in the upper panel starts with ro rate, but at the arrow suddenly increased out rate. In the lower panel the broken line th a fairly well preserved flow (0.94 ml/ g), changes to zero then regains the initial for another 30 before a second period with flow until finally the hydrogen gas is washed at a rate of 1.08 min

he last washout curves shown in panel 3 B were le 32 min after release of the tourniquets, and wed slow saturation of H in all areas. One area rked with solid line bottom panel) was prob- saturated only by diffusion from neighbour s with higher H concentration and continued a saturation for long period. The washout rates n all sampling sites were low until i. infusion 19 saline was started (20 ml/min). After a few utes the washout rates increased at different sampling sites at different time as marked with arrows.

Correspondingly the electromagnetically meas- ured flow was also close to zero and increased concomitant with local flow when saline was in- fused. When infusion started Hct was 68% indicat ing a fast severe plasma fluid loss after release of tourniquet.

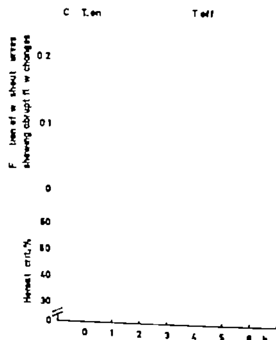


Fig. 4. Average hematocrit (below) and fraction of single washout curves within each hour showing abrupt flow changes (above) in control (C) after application (T on) and after release (T off) of the tourniquets.

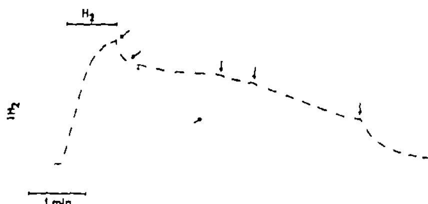


Fig 5 H_2 washout from 2 single electrodes about 60 min after phenoxybenzamine administration showing several changes in washout rate (arrows)

Patchy intermittent flow changes were totally observed in all except 1 animal in 7 dogs only 2–15 changes on 1–2 electrodes but in 7 dogs 15–45 times irregularly from different sampling sites The frequency increased markedly after release of the tourniquets The average fractions of washout curves showing abrupt flow shifts within each hour were about 0 2 in the 2nd and 3rd h after tourniquet release (Fig 4)

Measurements 2 h after release of the tourniquets

AP RAF and local cortical blood flow continued to fall througout the whole experimental period so that 2 h after tourniquet release the AP RAF OCI and ICF were reduced to 74 43 32 and 41 % of control respectively (Fig 1) OCI 1 17±0 47 ml/min g was not significantly different from ICF 1 23±0 57 ml/min g

The total plasma protein concentration increased to 6 32±1 1 mg/100 ml plasma i e 21 % above control while Hct increased to 58±5 % i e 57 % above control This shows that the plasma fluid loss was accompanied with leakage of plasma protein

Abrupt shifts in rate of washout were still observed after more than 2 h but less frequently as blood flow decreased to very low levels Before ending 5 experiments the dogs were given 0 –0 8 l of saline 1 dog even within the h period after tourniquet release (Fig 3B) to restore the AP and blood flow Saline infusion increased renal blood flow rapidly

In 5 experiments the α receptors in the renal vascular bed were blocked by phentolamine or phenoxybenzamin at a time with frequent shifts in local cortical flow on one or more electrodes Complete block was tested with i a injections of noradrenaline This block did not prevent the appearance of sudden changes in local flow as shown in Fig 5 where one washout curve has 5 different washout rates and the other 2 rates close to one hour after treatment RAF increased slightly after α receptor blocking agents

Further heparinization of 5 dogs did not prevent appearance of vascular shifts suggesting that microthrombosis cannot be the explanation of the intermittant flow

DISCUSSION

The local cortical blood flow obtained in control well within normal range previously obtained with the hydrogen washout method (Aukland et al 19 Tyssebotn & Kirkebo 1979) and well correlated the total renal blood flow measured with electromagnetic flowmeter

Sympathetic nerve activity after tourniquet application

Measurements performed few min after the compression showed transitory hyperventilation increased AP and slightly decreased renal perfusion. This compatible with an increased nervous tone in the renal vascular resistance Trueta et al (19 described a reduction of the renal artery caliber immediately after tourniquet application in rabbits concluding that this vasoconstriction was caused by neurovascular reflexes initiated in the injured limb This agrees with the findings of Barnes & Tr (194) that a tourniquet applied to one limb cause a vascular spasm in the contralateral and that denervation prevented the changes in Rowell (1979) concluded recently that the response was mediated by c fibers from the occluded limb man

-d on angiographic studies, Trueta et al also described diversion of intrarenal blood rom superficial to juxtamedullary glomeruli pplication of a tourniquet or by stimulation of atic or splanchnic nerves.

similar redistribution was reported by anz et al. (1968) using [85]Kr clearance during hnic nerve stimulation or ligation of the l arteries. On the contrary in the present e found that OCF and ICF were reduced in me proportion. Likewise bilateral occlusion carotid arteries in conscious dogs increased ascular resistance by about 40% thus indi-some reflex sympatic activity but the OCF/ atio remained unchanged (Kirkebo & Tys-n 1974). However Aukland (1976) has dem-ated that multicomponent analysis of the clearance curves gives unrealistic zonal flow and volume fractions.

t local cortical blood flow often changed from mogeneous flow pattern to an uneven and ping distribution of cortical blood flow W previously observed a similar pattern when ad increased to above 55% during dehydra-(Kirkebo & Tyssebotn 1977) or in endotoxin k (Kirkebo & Tyssebotn, 1980). In the pres-tudy the abrupt shifts were observed shortly tourniquet application when the Hct could im-ably have increased to high levels, since Hct at end of 4 h of occlusion only had reached 47%.

creased sympathetic nerve activity could pos-y increase Hct by a reflectory contraction of the en, pushing packed red cells into the circula-nce 150 ml red cells would be sufficient to unt for the observed rise in Hct during occlu-However at the same time the increased etone and/or increased catecholamine blood l ould drain fluid from the unaffected muscles thereby tend to reduce Hct (Mellander & Jo-sson, 1968)

urther even though the slings around the limbs n tightened as firmly as possible some blood y have passed through the arteries into the luded limbs. On the other hand, the hindlimbs re not usually seen to swell until release of the ngs, so that only negligible amount of blood could l behind the slings during the 4 h occlusion and id not cause the generally observed increase of t.

The patchy intermittent flow in the early phase y have been mediated by nervous reflexes concomitant with hyperventilation and a rise in AP An explanation based on circulated vasoactive hormones is not probable considering the prompt occurrence after tourniquet application and that infusion of vasoactive substances in the renal artery caused a homogeneous change in cortical blood flow (Tyssebotn & Kirkebo 1975 1979)

However when the tourniquet was thightened some tissue on the proximal side may be damaged either by direct trauma or by ischemia. Toxic products could in time enter the general circulation eventually by leakage beyond the tourniquet or from the damaged tissue and possibly cause some change in total or local renal blood flow Such explanations are not likely for the flow shifts during the early phase of hindlimb occlusion but cannot be ruled out during the late phase of the 4 h occlusion period

Heterogeneous cortic l blood flow after releas of the tourniquet

The dogs started again to hyperventilate immediately after release of the tourniquet, probably due to acidic/toxic metabolites entering the systemic circulation from the cooled and damaged hindlimb tissue This explanation is supported by Liu (1971) who found the same responses in untraumatized dogs when cross circulating blood from dogs traumatized with hindlimb occlusion of comparable duration to that in our animals. These released substances lead to an abnormal leakage of fluid from the capillary bed causing edema and Hct elevation As the occluded legs again were circulated the hindlimbs started to swell profusely

Takács & Kalláy (1957) found the same changes in blood flow whether denervating the kidney or not, concluding that the circulating agents caused a vasoconstriction. With a Hct of 58% as in the present study it is doubtful whether the renal vascular hindrance was increased, since the iscosity rise is probably greater than the calculated increase in resistance (Schmid-Schonbein 1976) However the vascular responses in outer and inner cortex were equal

At high Hct and decreased AP the frequency of intermittent patchy flow distribution increased as previously described (Kirkebo & Tyssebotn 1977 1980) However since patchy intermittent flow also w s seen before Hct was increased, high Hct cannot be the only explanation for patchy flow but most lik ly facilitates the observed changes

During development of shock the plasma leakage continued and the total renal perfusion could eventually cease (Fig 3B) However the situation was reversible as dilution of the red cells by saline infusion reopened the vessels and both total and local flow increased rapidly after few min of infusion This must mean that permanent thrombi were not formed at this state indicating that local clotting of blood in limited areas is not a likely explanation This is further supported by the findings that heparinization with adequate doses did not prevent the occurrence of patchy flow changes

Hansson (1965) has shown that the kidney is vulnerable to thrombocyte aggregation induced by administration of crushed connective tissue suspension or ADP Starting with the same procedure Jorgensen et al (1970) observed later patchy tubular necrosis and focal glomerular nephritis On the other hand Collan & Alho (1973) found no microthromboses of platelets by ultramicroscopy until 24 h after opening of tourniquets

An uneven distribution and affinity of α receptors responding to nerve activity or circulating catecholamines could possibly explain the patchy cortical flow but since the α blocking agents did not reduce the occurrence of blood flow shifts this could not be the only reason One may suspect tha local agents might play a role in provoking the phenomenon

In conclusion OCF and ICF were proportionally reduced during tourniquet shock The occurrence of patchy intermittent cortical ischemia is probably facilitated by a high Hct during shock but was also seen before Hct did rise α receptor blocking or heparinization did not abolish the phenomenon

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cts of glucagon and pentagastrin on glibenclamide-induced lin release

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AHRÉN B & LUNDQUIST L. Effects of glucagon and pentagastrin on glibenclamide induced insulin release. Acta Physiol Scand 1980, 109 261–264 Received 8 Nov 1979 ISSN 0001-6772. Department of Pharmacology University of Lund Sweden.

It was recently reported that administration of sulphonylureas may lead to stimulation of the release of glucagon and gastrin-like peptide These peptides may then eventually influence the insulin releasing action of the sulphonylureas. Therefore the effects of glucagon and pentagastrin on basal and glibenclamide-induced insulin secretion were studied It was found that at dose of 4.25 nmol/kg the two peptides induced a significant stimulation of basal insulin secretion. However pentagastrin at the dose of 4.25 nmol/kg did not influence the insulin release induced by glibenclamide, whereas an equimolar dose of glucagon potentiated the insulin secretory response to this sulphonylurea drug by about 40% Glucagon in contrast to pentagastrin thus positively modulates the insulin secretory pathway stimulated by the sulphonylurea drug glibenclamide

Key ords. Glucagon, pentagastrin, glibenclamide, insulin secretion

lphonylureas used in the treatment of insulin ndent diabetes have been shown to stimulate lease of insulin (Loubatières 1969). Also the e of other hormones such as glucagon and a may be influenced by these drugs The gon release has been reported to be stimulated atières et al 1974) inhibited (Samols, Tyler Ike 1969) or uninfluenced (Kajinuma, Kuzuya 1974) by sulphonylureas. Later it was shown n the rat tolbutamide in high concentrations ts glucagon release whereas in low concenrts it stimulates glucagon release (Grodsky et 977). Recently it was reported that in the cat onylureas also stimulate the release of imreactive gastrin of antral as well as of neural (Uvnäs-Wallensten et al. 1979)

sulphonylureas stimulate the release of glun and if the reported immunoreactive gasreleased by sulphonylureas shares biological ity with authentic gastrin the effects of gon and gastrin on sulphonylurea-induced inrelease might be of importance In the present y the in vivo effects of glucagon and pentagason insulin release stimulated by the sulylurea drug glibenclamide were investigated in mouse.

METHODS

Drugs

Pure porcine glucagon was obtained from Novo Research Institute, Copenhagen, Denmark. Pentagastrin (Peptavlon®) was from I C I Ltd. Macclesfield, Cheshire, Great Britain and glibenclamide from Boehringer Mannheim GmbH, Germany The peptides were dissolved in saline with addition of 0.1% gelatine.

Animals

Female mice of the NMRI strain (Laboratory animal Breeding, Laven, Denmark) weighing 25–35 g, were used. The animals were fed standard pellets (Astra-Ewos, Södertälje, Sweden), and tap water ad libitum before and throughout the experiments.

Experiments

1 *Effects of glucagon or pentagastrin on basal insulin release* Each of the 2 peptides was injected in tail vein, 4.25 nmol/kg, 10 µl/g b wt. was injected Control animals were injected with saline. Two minutes after the injection 250 µl blood was drawn by orbital puncture. The mice were unanaesthetized throughout the experiments.

2. *Effects of glucagon or pentagastrin on glibenclamide-induced insulin secretion* Each of the peptides was injected 5 µl/g, 15 prior to rapid injection of 5 µl/g glibenclamide Controls were injected with saline and glibenclamide, or with saline alone. min after the last injection blood was collected. This sequence of the injections was chosen in order to mimic the anticipated glibenclamide-induced humoral insulinotropic ef-

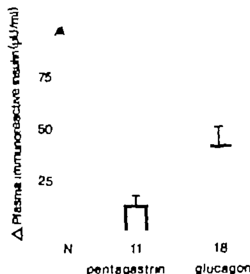


Fig 1 Increase in plasma immunoreactive insulin following the intravenous injection of 4 25 nmol/kg body weight of pentagastrin and glucagon respectively Blood sampling was performed 2 min after the injection Bars indicate standard errors of the mean N = number of mice

fects since the peptides released by oral intake of sulphonylurea may reach the insulin cells before the drug Repeated experiments in this laboratory have shown that the maximal concentration of immunoreactive insulin in mouse plasma following a rapid i injection of glibenclamide is achieved after 1.5—.5 min

Glibenclamide was administered in a dose giving about 50% of its maximal insulin response This dose was 62 5 μg/kg Glucagon and pentagastrin were administered at a dose of 4 25 nmol/kg

Determination of insulin

The concentrations of immunoreact ve insulin (IRI) in plasma were determined by a radioimmunoassay technique (Heding 1966) using ^{125}I labelled pig insulin and guinea pig anti-pig insulin

RESULTS

Effects of glucagon or pentagastrin on basal insulin release

Each of the 2 peptides was injected i v 4 25 nmol/kg and 2 min later blood was collected Control animals were given saline It was found (Fig 1) that glucagon enhanced the plasma IRI concentrations by 42.3±9 5 μU/ml above the controls (P<0 001) whereas pentagastrin induced an elevation of the plasma IRI concentrations by 13 2±3 9 μU/ml (P<0 01).

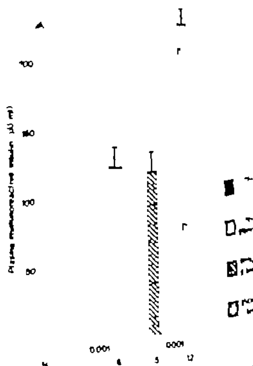


Fig 2 Effect of pentagastrin and glucagon, respectively on insulin release stimulated by glibenclamide (open column) pentagastrin (4.25 nmol/kg) (hatched column) or glucagon (4.25 nmol/kg) (stippled column) injected intravenously 15 s prior to glibenclamide μg/kg) Blood sampling was performed 2 min after the glibenclamide injection Control animals received saline (black column) Bars indicate standard errors of the mean P = probability level of random difference n s = not significant. N = number of mice

Effects of glucagon or pentagastrin on glibenclamide-induced insulin release

Glibenclamide 62.5 μg/kg injected i v increased the plasma IRI concentrations to 124 1±15 4 μU/ml (P<0 001 vs the saline injected controls) (Fig 2). Glucagon 4.25 nmol/kg was injected 15 s prior to glibenclamide and it was found that the plasma IRI concentrations were further increased to 226 2±11 4 μl/ml (P<0 001 vs. the glibenclamide control group) Since glucagon exerted a stimulatory effect on basal insulin secretion by itself expected concentrations of plasma IRI following the injections of glucagon plus glibenclamide can be calculated to be about 160 μU/ml The observed plasma IRI concentrations in the group treated with glucagon plus glibenclamide 226.2±11 4 μU/ml are significantly higher than this expected value (P<0 001) Thus it was found that glucagon potentiated glibenclamide induced insulin release by about 40%

tion of pentagastrin, 4.25 nmol/kg plus benide resulted in a mean plasma IRI conion of 119.6±15.2 μU/ml (n.s. vs the gliamide control group) Thus, contrary to glupentagastrin did not affect glibenclamide d insulin release.

USSION

stimulates the release of insulin (Rehfeld of Fig. 1) It may function as an intrapanregulator of insulin release since it may be creatic neural (Larsson & Rehfeld 1979) or atic neonatal endocrine (Larsson et al 1976) Furthermore, immunoreactive gastrin of and neural origin in the cat was recently strated to be released by glibenclamide (Uvallensten et al. 1979) If this immunoreactive possesses bioactivity gastrin might be of ance in mediating insulin releasing effects of phonylurea drugs. This could be a parallel to ported glibenclamide-induced release of a al factor with insulin releasing properties atten et al. 1977). However we failed to obany influence of pentagastrin on glibenide-induced insulin release This does not ole important effects of gastrin in mediating actions of glibenclamide.

cagon has earlier been shown to stimulate the e of insulin (Samols, Marri & Marks 1965 per & Orci 1976) This study shows that ry to pentagastrin glucagon potentiated clamide-induced insulin secretion. A syner between glucagon and tolbutamide with regard lin secretion in man has earlier been reported trom & Cerasi 1977) Such synergism might importance in sulphonylurea treatment of tes if these drugs stimulate the release of gon. Moderately increased circulating levels cagon in diabetes (Unger 1978, Lefebvre & lx 1979) may also make sulphonylurea drugs powerful in stimulating the release of insulin cose and sulphonylurea drugs show some ences in their insulinotropic effects Me nes and fluxes of calcium (Malaisse Brisi & Baird 1973 Malaisse, Pipeleers & Mahy l handling of cAMP (Grill & Cerasi 1973 Sams netagne 1974) lysosomal activation (Lundquist Lundquist 1975) and intracellular amines rdquist, Ekholm & Ericson 1971 Lundquist Ericson, Håkanson & Lundquist 1977) seem all to be of importance in a similar way for these secretagogues Recent studies in our laboratory (unpublished) indicate that glucose-stimulated insulin release in mice in potentiated by glucagon but not by pentagastrin which further shows the similarity between glucose and glibenclamide in their insulin releasing activity Glucagon in contrast to pentagastrin thus may positively modulate some events of importance for the insulin-releasing activity of both glucose and glibenclamide

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ultaneous measurements of capillary filtration diffusion capacities during graded infusions oradrenaline (NA) and 5 hydroxytryptamine (5–HT) the rat hindquarter vascular bed

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RIPPE B & FOLKOW B Simultaneous measurements of capillary filtration and diffusion capacities during graded infusions of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) into the rat hindquarter vascular bed. Acta Physiol Scand 1980 109 265–273 Received 8 Nov 1979 ISSN 0001-6772. Department of Physiology University of Göteborg, Sweden

The relationships between capillary diffusion capacity (PS) for Cr EDTA respective capillary filtration capacity (CFC) and vascular resistance during graded intraarterial infusions of NA and 5-HT into the artificially constant flow perfused rat hindquarter vascular bed were investigated During maximal vasodilatation PS for Cr-EDTA was some 5.5–5 7 ml/min 100 g, CFC some 0 04 ml/min mmHg × 100 g, while vascular resistance was 2.8 mmHg × ml⁻¹ × min 100 g (PRU_{100}) and isogravimetric capillary pressure 12.8 mmHg on an average Setting out from maximal vasodilatation, increasing doses of NA and 5-HT produced graded reductions in capillary surface area as reflected by progressive decreases in both PS for Cr EDTA and CFC These changes occurred simultaneously with progressive increases in both pre and postcapillary resistances, causing elevations in both arterial and capillary hydrostatic pressures and hence in capillary fluid filtration at constant flow Capillary hydrostatic pressure increased maximally to 45 mmHg (calculated for NA) and vascular resistance to some 21 mmHg ml⁻ min 100 g on an verage. PS for Cr-EDTA decreased maximally to some 0 7 1 ml/min 100 g for both NA and 5-HT and furthermore the relationships between PS for Cr-EDTA and PRU_{100} for NA respective 5-HT ere almost identical This was taken to indicate that capillary surface area for nutritional xchange is affected similarly by both drugs. However the CFC-PRU_{100} relationship w shifted towards some 30-50% higher CFC values for 5-HT than for NA at almost every level of vasoconstriction. This might suggest that 5-HT besides reducing capillary surface area also induces moderate increases in capillary permeability through increases in number and/or radius of large pores (gaps) (cf Rippe Kamiya & Folkow 1978). E en during NA-induced vasoconstriction, when virtually no changes in capillary permeability occurred PS for Cr EDTA was reduced to relatively greater extent than CFC the discrepancy being most pronounced during marked vasoconstriction The significance of this finding is discussed

Key ords Capillary permeability indicator diffusion, capillary filtration, isogravimetric capillary pressure, rat, noradrenaline, 5-HT

earlier study (Rippe, Kamiya & Folkow 1978) lary filtration and diffusion capacities were fol- d simultaneously in the maximally dilated rat quarter ascular bed over wide range of in- d changes in capillary permeability-surface product (PS) This study included a rarification e capillary network by means of i.a. injected ospheres (Z≈15 µm) to create a table model ation for the in vivo events when precapillary oth muscle constriction reduces the perfused capillary surface area ("precapillary sphincter function") The diffusion capacity (PS) for Cr EDTA, measured with a colorimetric on line variant of the single injection indicator diffusion method (Rippe & Stage 1978) then decreased largely in proportion to the increased resistance to flow after microvascular plugging while capillary filtration coefficient (CFC) decreased less It was concluded that the induced type of precapillary plugging implied a capillary flow pattern that ranged from complete

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74) were performed. Isogravimetric capillary pressure (P_{ci}) was determined as described earlier (Eliassen et al. 1974, Kamiya, Rippe & Folkow 1979). NA or 5-HT was administered in increasing concentrations in 5 to 6 steps until maximal vasoconstriction was reached as indicated by maximal P values. Care was taken to make the infusion periods as short as possible, every step being of about 4 min duration in order to avoid too much of edema formation in the preparation, as a result of the drug-induced postcapillary vasoconstriction which corresponds to elevated P in the constant-flow situation. During each step, changes in CFC, PS and capillary fluid equilibrium were recorded and double determinations of CFC and PS were performed as far as possible.

In 5 expts. NA and 5-HT were given consecutively in the same preparation, the sequence between them being alternated. Increasing doses of either NA or 5-HT were given in 3 plus 3 expts. In an additional series of 4 expts. supramaximal doses of NA was administered immediately after the control period.

PS determinations were performed as described earlier (Rippe & Haraldsson 1978, Rippe, Kamiya & Folkow 1978). The PS value was calculated according to the formula $PS = -(Q-J/2) \ln (1-E)$, where Q is the directly measured perfusate flow and J the filtration rate while E is the extraction fraction. Area extraction (E_{area}) up to the time when the tracer concentrations had declined to around 5% of peak values was used for the calculations. In the present study the convectional contribution to total solute transport was slightly underestimated. The reason is that no correction was made for transcapillary loss of the reference tracer in the present study. At a filtration rate of 0.3 ml/min × 100 g the convectional contribution to total transport has been determined to 5–6% (Rippe, Kamiya & Folkow 1978) of total transport which means that total solute transport was underestimated by some 3–5%.

RESULTS

NA and 5-HT effects on pre- and postcapillary resistances

During control before the start of the drug infusion, P was 37.5±0.5 mmHg (mean ± S.E.), P was ... 0.4 mmHg, perfusate flow (Q) 1... 3±0.3 ml/min × 100 g and PRU_{tot} ...8+0.1 mmHg×min×ml^{-1} × 100 g for the whole material. Isogravimetric capillary pressure (P) during control was estimated to ...4 mmHg which agrees with earlier P_{ci} determinations on this preparation during dextran-horse serum perfusion (Kamiya, Rippe and Folkow 1979). The relation between pre- and postcapillary resistance during control was in this study around ...1 which is slightly higher than during pure dextran perfusion (around ...1, Folkow et al. 1977). The reason may partly be that the precapillary smooth muscle cells are more difficult to keep fully relaxed when horse serum is present in the dextran perfusate, partly that capillary permeability characteristics are slightly different as reflected by a higher CFC value (around 0.05) and a lower P (around 1 mmHg) when dextran Tyrode is used.

As flow was kept constant throughout each experiment and also relatively unchanged from experiment to experiment (11.5–14.0 ml/min × 100 g) changes in (P_A–P) during drug infusion closely reflect changes in total resistance to flow. In Fig. 1 (P–P) values are plotted against the dose of NA or 5-HT. The dose-response curves are almost identical to those obtained in earlier studies from this group (e.g. Folkow et al. 1977, Åblund et al. 1977). During every dose step the rate of capillary filtration was recorded as well.

In Fig. ... filtration rate is plotted against PRU_{tot}. Provided that capillary permeability and tissue hydrostatic and colloid osmotic pressures remain essentially constant, the drug-induced P increases (above P_{ci}) can be assessed by dividing the filtration rate by the prevailing CFC value (Folkow et al. 1974, Eliassen et al. 1974). As it could be suspected that 5-HT affects capillary permeability as well (see below), P determinations and hence determinations of pre- and postcapillary resistances were performed only on the NA data. Such an analysis is shown in Fig. 3. During NA induced maximal vasoconstriction P increased to ...57.3± 5.3 mmHg and P to 41.4±2.4 mmHg, while P was kept constant at ...8±0.4 mmHg. As mentioned above P_c during maximal vasoconstriction was obtained under the assumptions that capillary permeability and tissue hydrostatic and osmotic pressures were largely unchanged during the course of the drug infusion. However, adding 5–10 ml of fluid to the interstitial space, which was the consequence of the drug-induced capillary filtration up to the level of maximal vasoconstriction, usually creates around 3 mmHg of absorbtive forces at the capillary level (Eliassen et al. 1974). This was also evident from 4 experiments in which supramaximal NA doses were given directly after the control period when filtration rates were some 10–15% higher than in Fig. 2 at maximal vasoconstriction. Thus, a P value around 45 mmHg seems more appropriate than the value given above. The corrected pre- to postcapillary resistance ratio at maximal vasoconstriction is then around 5/1, which closely agrees with the value given by Folkow et al. 1977. The moderately higher P and P values obtained in

flow cessation in some capillaries via various degrees of reduced flow in capillaries fed by collaterals up to a situation of unimpeded flow in nearly all capillaries In such a situation PS should be more reduced that CFC since it is more affected by flow limitation and heterogeneity than is the filtration process

Towards this background it was considered of importance also to investigate the impact on PS for Cr EDTA CFC and capillary fluid equilibrium of graded vasoconstrictions induced by infusion of noradrenaline (NA) and 5-hydroxytryptamine (5 HT) which in the prevailing situation of constant flow perfusion ought to produce a sufficiently well maintained pattern of microvascular constriction to allow for repeated measurements Such an investigation is of particular interest since existing data in this field show considerable disagreement (cf Haddy Scott & Grega 1976) Thus infusion of noradrenaline at constant flow perfusion of dog skeletal muscle has been reported to either decrease ^{86}Rb uptake (Szwed & Friedman 1975) or to increase it (Gable et al 1964) while CFC appeared to remain largely at control after a transient decrease during similar conditions (Kaiser & Duana 1974) In contrast during in vivo constant pressure conditions CFC is reported to increase beyond resting control during noradrenaline infusion (e g Järhult 1971) while ^{86}Rb uptake is reported to decrease (Appelgren & Lewis 1968) However in none of the mentioned studies CFC and PS were measured simultaneously and it is therefore impossible to judge whether the apparent descrepancies between the changes in CFC and PS are due to real differences between the two capilary exchange parameters or simply reflect methodological differences and/or different patterns of vascular responses due to the prevailing experimental situation

Also concerning the effect of 5 hydroxytryptamine (5-HT) on transcapillary exchange processes there are some controversies perhaps also due to the fact that 5 HT effects on capillary permeability might be species dependent (cf Haddy Scott & Grega 1976) Thus there is evidence that 5 HT increases capillary permeability in rat (e g Rowley & Benditt 1956 Majno Palade & Shoefl 1961 Gabbiani Badonnel & Majno 1970) while it seems to be largely unaffected in dog (e g Merill et al 1974) rabbit (Ebert & Graham 1966) and in man (Glover et al 1958 Roddie Shephard and Whelan 1955)

In the present experiments the effects of graded infusions of NA and 5-HT on PS for Cr EDTA CFC and capillary fluid equilibrium were studied [illegible] the initially maximally vasodilated vascular bed of rat hindquarters during well controlled [illegible] dynamic conditions and at constant flow Earlier studies in which capillary diffusion and filtration capacities were measured simultaneously [illegible] Rippe Kamiya & Folkow 1978 Rippe & [illegible] 1978) indicate that such an approach can provide detailed information about drug induced changes [illegible] capillary permeability-surface area product

METHODS

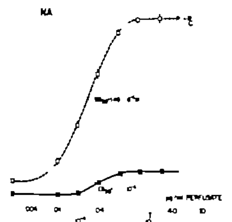
Experiments were undertaken on the isolated hindquarters from 16 male Wistar albino rats weighing between [illegible] and 380 g The preparations were arranged for the gravimetric technique (Pappenheimer & Soto Rivera [illegible]) and for a colorimetric on line variant of the [illegible] tion method (Rippe & Stage 1978).

The preparation as well as the general technical arrangements have been described earlier (Rippe, [illegible] Folkow 1978) Briefly the hindquarters were isolated [illegible] tight mass-ligatures at a level between the renal and [illegible] lumbar vessels and the spinal channel and bone [illegible] were plugged with cotton wool soaked in [illegible] leakage from vessels or tissue was accepted [illegible] paws were excluded by ligatures so that about [illegible] sisted of skeletal muscle the remainder being [illegible] adipose tissue and bone

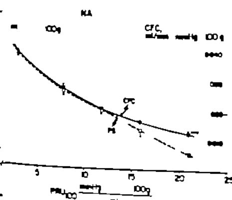
The preparation was artificially perfused at constant flow via the aorta by means of Harvard perfusion [illegible] Arterial pressure (P) was measured continuously [illegible] tail artery and venous outflow pressure (P) in one of [illegible] renal veins The completely isolated hindquarter was continuously weighed on a balance plate connected to a [illegible] gauge and tissue weight as well as pressures were monitored on a Grass polygraph The venous outflow cannula draining the caval vein was couples in series with [illegible] meter and a densitometer system for simultaneous [illegible] ings of Cr EDTA and Cardio-Green bound to [illegible] These indicators were used f measurements of [illegible] diffusion capacity (Rippe & Stage 1978). pH and the [illegible] tion curves were monitored on a potentiometer [illegible] (Rika Denki model B34) The free end of the [illegible] outflow cannula could be adjusted to any desired level [illegible] set the venous outflow pressure P Flow and P [illegible] controlled by adjusting the setting of the perfusion [illegible]

4 per cent dextran (Macrodex Mw about [illegible] AB Pharmacia Sweden) in Tyrode solution was [illegible] perfusate to which wa added horse serum ([illegible] Serum SBL Sweden) 100 ml/l The perfusate was [illegible] with a mixture of 97 % O_2 and 3 % CO_2 and kept at [illegible] Flow was kept constant at 11 5-14 (mean [illegible]) ml [illegible] 100 g throughout the experiment During control [illegible] vasodilatation was induced by slug injections [illegible] time) of papa erine

In the control situation three measurements of PS [illegible] Cr EDTA (Rippe & Stage nd of CFC (Flow [illegible]



Precapillary pressure drop (broke line) and post-ry pressure drop (solid line) vs. dose of NA (cf Fig part) As in Fig 1 alues on ED_{50} are given in the



CFC (solid line) and PS for C -EDTA (broken line) eripheral ascular resistance in the constant flow sed rat hindquarter vascular bed at different level of nduced asoconstriction Mean values ± S E. (9) 'control' (maximal vasodilatation) and at PRU_{100} of 1, 16 respectiv 21 mmHg min 100 g are given in the figure The dotted line demonstrates the parameters ould alse if there ere direct portionality) between increase in ascular resistance decrease in capillary surface area from the state of mal vasodilatation ('control') The relativ changes in and PS-C EDTA follow ach other closely hen ced to around one third of control but at further PS then the simultaneous CFC changes are relatively ler During maximal asoconstriction CFC reduces to and 25% of control and PS-C EDTA to about half alue the differenc being statistically highly sig ant (P 0.001)

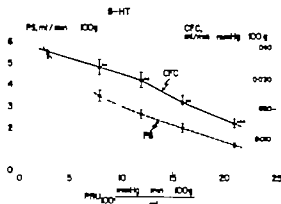


Fig 5 CFC and PS-C -EDTA plotted against peripheral resistance at different levels of 5-HT-induced vasoconstriction. Mean alues ± S E. (=9) at the same PRU_{100}-levels as in Fig. 4 are show Statistical comparison of CFC with PS-Cr-EDTA at every 'test'-level of PRU_{100} was performed by dividing the test values by 'control' value (i.e that during max vasodilatation) and utiliz ing these relative values in Student t-test for group comparisons It was then evident that the curve representing CFC alterations is significantly displaced upwards in comparison ith that representing PS alterations ($P<0.01$) The last mentioned curve almost identical to that for NA, while the curve representing CFC changes is displaced (30–50%) upwards in relation to that for NA (cf Fig. 4)

3 *Simultaneous change in CFC and in PS for C EDTA during 5-HT infusion*

The relationship between changes in CFC and PS for Cr EDTA and total vascular resistance during 5-HT infusion are shown in Fig. 5 The curve repre senting the PS changes is almost identical to that for NA in Fig. 4 while the curve representing the CFC changes is displaced (30–50%) upwards in relation to that for NA and this change is statistically significant ($P<0.01$) The PS-changes ranged from 5 7 to about 1 0 ml/min × 100 g while the CFC changes ranged from 0 04 to about 0 015 ml/min mmHg × 100 g.

DISCUSSION

The actions of NA and 5-HT on transcapillary fluid and solute exchange are partly controversial (Haddy Scott & Grega 1976) perhaps reflecting the difficulties involved i capillary exchange studies I an earl er study where filtration and diffusion event were simultaneously measured (Rippe Kamiy & Folkow 1978) it wa shown that qualita

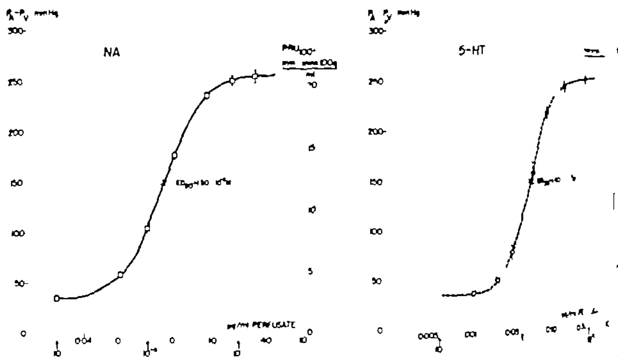


Fig 1 Average constant flow perfusion pressure plotted against concentration of NA (left part solid line) and 5-HT (right part broken line) Total peripheral vascular resistance expressed in PRU ₁₀₀ is also indicated in the figure (right hand ordinates) Mean values ± S E from 9 expts with NA (squares) and 9 with 5-HT (circles) are shown. The concentration of either drug effective of producing 50 % of maximal pressor response is denoted ED_{50}

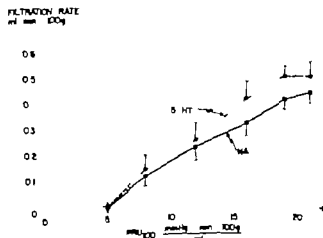


Fig 2 Filtration rate vs peripheral vascular resistance during infusion of increasing doses of NA (solid line) and 5-HT (broken line) during constant flow The increase in capillary filtration with increasing PRU_{100} and drug concentration during constant flow is most likely due to a progressive increase in postcapillary resistance and hence in Pc (cf Fig 3) As regards 5-HT increases in capillary permeability producing decreases in effective transmicrovascular colloid osmotic pressure gradient might also be taken into account in explaining the data Though 5-HT seemed produce higher filtration rates than NA at every level of increased peripheral resistance the difference is not statistically significant

the present study compared with that of Folkow et al 1977 could be explained by the [illegible] flows

Simultaneous changes in CFC and PS for Cr EDTA during NA-infusion

In Fig 4 the simultaneous changes in CFC and PS for Cr EDTA during NA infusion and constant flow conditions (flow 11 5–14 ml/min × 100 g) are plotted against total vascular resistance expressed in PRU ₁₀₀ units It is seen that the relative changes in CFC and PS closely follow each other when reduced down to one third of controls by vasoconstriction but at further PS reductions the simultaneous CFC changes are relatively small Further the NA effects on the true resistance vessels are initially more pronounced than the precapillary sphincter vessels when expressed as reduced PS and CFC In case the two effects had run parallelly the relation between CFC-PS and PRU ₁₀₀ would have followed the dotted line in Fig 4 From the state of maximal vasodilatation to maximal vasoconstriction PS ranged from [illegible] ml/min × 100 g (8 times decrease) while CFC fell from about 0 04 to 0 01 ml/min × mmHg × 100 g (4 times decrease)

ow far more reduced than is CFC This latter on resembles that obtained during precapil- lugging with microspheres (Ø~15 μm) (Rip- amiya & Folkow 1978) which reduces PS sub- lly more than CFC This response pattern to be due to the fact that substantial filtration ll occur in capillaries perfused at very low bile diffusion exchange is here severely d by flow limitation

eneral these results in a well controlled con- low situation in vitro are in agreement with o results by Lundvall & Järhult (1976). vasoconstrictor fibre stimulation to the cat ub muscle ascular bed they found evidence drenergic dilatation of precapillary sphincter s paralleling the α-adrenergic constriction of terolar segments to some extent also noted NA infusions (cf Järhult 1971).

oretically the observed discrepancy between d CFC changes during marked NA vaso- nction might also reflect increases of capil- ermeability caused by the capillary pressure ses consequent to the constant-flow situation. ver in the rat hindquarter vascular bed pres- nduced increases in capillary permeability oc lnst at capillary hydrostatic pressures well 45–50 mgHg (Rippe Kamiya & Folkow 1979) uch a mechanism would therefore hardly af he measurement

ring 5-HT nfusion the relationship between ductions and resistance increases was almost ical to that during NA-induced vasoconstric while the CFC reductions were displaced 30- upwards compared with the situation during vasoconstriction This may reflect a different triction pattern on the precapillary essels for T than for NA and/or that 5-HT incr ses lary permeability At high 5 HT concentration is evidence of a relative decrease in small el precapillary resistance in dog forelimb mus- herens mall essel resistance n the skin in- ses markedly (Merill et al 1974). I the rat dquarter preparation mainly consisting of etal muscle (?1?), the possibility of a phinc dilatation in response to 5-HT paralleling the ked resistance increases cannot be completely d out The main mechanism however behind relatively higher levels of CFC during 5-HT sion is likely to be that 5-HT increases capillary meability in rat well documented in this ces (e g Rowley & Benditt 1956 Sparrow & Wilhelm 1957 Majno & Palade 1961 Majno Gilmore & Leventhal 1967) This permeability increase seems to reflect a formation of venular gaps (large pores cf Grotte 1956) probably by means of endothelial cell contraction causing partial detach ment of intercellular junctions (Majno Gilmore & Leventhal 1967 Majno Shea & Leventhal 1969) Further evidence for such a mechanism has recent ly been obtained also for histamine in various ex periments (eg. Rippe & Grega 1978)

Maximal 5-HT increases in capillary permeability were obtained at 5-HT concentrations around 0 05–0 08 μg/ml (1.2– $\times 10^7$ M) a indicated by the CFC data in Fig. 5 compared with those in Fig. 4 Thus on a molar basis 5-HT seems to be at least 100 times more potent than histamine which in the rat is effective in the concentration range of 3–20 μg/ml (~ .7–18$\times 10^5$ M Rippe & Grega 1978) in agreement with earlier studies (Sparrow & Wilhelm 1957) On the other hand the 5-HT induced CFC increase above that during NA infusion was only some 30–50% which is 5–8 times lower than the maximal CFC changes evoked by histamine at a constant capillary surface area (cf Rippe Kamiya & Folkow 1978) It is in line with the comparatively weak permeability increasing effects of 5-HT re ported by Majno Gilmore & Leventhal (1967) who studied vascular permeability after local applica tions of bradykinin histamine and large doses of 5-HT (100 μg/ml) to the rat cremaster muscle Assuming a rough proportionality between CFC changes and increased large molecular permeability by 'histamine type mediators a 50% CFC increase would mply a decrease in effective transcapillary colloid osmotic pressure difference of at most –3 mmHg (cf Rippe Kamiya & Folkow 1978). Thus most of the ncreased fl id filtration caused by 5-HT during the constant flow condition in this study s likely to be caused by capillary pres ure eleva- tions due to postcapillary vasoconstriction and only a small fraction due to pressure-independent mechani ms (cf Kline et al 1974)

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tively different exchange parameters can respond quite differently for given changes in either capillary permeability or surface area Thus histamine induced permeability changes were generally well reflected by changes in capillary filtration coefficient (CFC) and capillary fluid equilibrium while capillary diffusion capacity for small molecules (PS) was very little affected Changes in capillary surface area were on the other hand closely mirrored by PS changes while CFC always changed less

Such discrepancies may be one of the main reasons for the inconsistencies between data from various laboratories concerning the effects of NA and 5-HT on capillary exchange It would therefore be of interest to study simultaneously principally different exchange parameters in the same experiment to gain further insight into these problems Towards such a background the present experiments were performed in which PS for Cr EDTA CFC and capillary fluid equilibrium were simultaneously measured during well defined haemodynamic conditions and at constant flow during graded infusions of NA and 5 HT into initially maximally vasodilated rat hindquarters

The NA induced relative changes in pre and postcapillary resistances (see Fig 3) agree with recent measurements in rat by Folkow et al 1977 though the absolute changes were moderately greater in the present study presumably because of higher flow rates For 5-HT the pre and postcapillary resistance dose-response curves seemed to be fairly similar to those for NA though the postcapillary resistance increase seemed to be less marked for 5 HT This was deduced from the filtration data in Fig 2 and the CFC data in Fig 5 under the assumption that 5-HT only induces minor Pc changes (up to 2–3 mmHg) during vasoconstriction which seems to be the case (see below)

NA infusion induced a dose related decrease in the effectively perfused capillary surface area as indicated by changes in both CFC and PS Thus CFC-changes from 0 04 ml/min × mmHg × 100 g during maximal vasodilatation to resting values around 0 013–0 015 ml/min × mmHg × 100 g (cf Cobbold et al 1963) were associated with essentially proportional decreases in PS from 5.5–6 to about 1 5 ml/min × 100 g (Fig 4) However at higher NA doses PS for Cr EDTA was further reduced down to 0 6–0 8 ml/min × 100 g while CFC decreased only to 0 011 ml/min × mmHg × 100 g This type of discrepancy has frequently been observed earlier though with the difference that the PS and CF measurements were performed in different experiments (cf Renkin 1969 Haddy Scott & Gr 1976) Thus in the constant flow perfused dog hindlimb (8–10 ml/min × 100 g) Kaiser & Dia (1974) noted that CFC first decreased from ... values during the first two minutes of NA infus and then generally returned to or slightly ab control In contrast Sz wed & Friedman (197.) du ing constant flow conditions (flow 3 ml/min × 10 g) observed a marked and sustained decrease i ^{86}Rb extraction in dog gracilis muscle during N vasoconstriction Also when NA infusions or va constrictor fibre stimulations cause flow redu tions during constant pressure conditions the seems to be marked discrepancies between CF and PS responses insofar as the former are ... reported to increase slightly (Cobbold et al 19 Järhult 1971) while the latter seem to decrease c siderably (Renkin & Rosell 196 Appelgren Lewis 1968)

Concerning the background of these discrep cies between PS and CFC responses during induced vasoconstriction Sz wed & Fried (1975) questioned the validity of CFC mea ments as an indicator of capillary surface area c pared with measurements of diffusion cap Renkin (1969) however suggested that the dis ancy rather reflects the complexity of the m vascular network where increases in precap resistance may give different CFC PS res patterns depending on which consecutive seg that are mainly constricted Thus progressive ure in an all-or none fashion of precapillary s ter regions would give proportional decre CFC and PS while more graded contr would change PS more than CFC since PS is flow dependent (Renkin 1969 Folkow & Mel 1970 Rippe Kamiya & Folkow 1978)

The present data suggest that NA vasocon tion from maximal vasodilatation to levels sponding to in vivo resting resistance vess where CFC values are around 0 015 ml/m ward & Diana 1978) implies a progressive ance increase coupled to a virtually compl striction also of sphincter segment that r the number of perfused capillaries some 3 However at further resistance increases the of CFC PS changes suggests a predomina constriction of segments affecting overall res more than the microvasc flow di tribution

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...ts of purified macrophage RNases on granuloma ...blasts with reference to silicosis

...) P LEHTINEN and E KULONEN

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AHO S LEHTINEN P & KULONEN E. Effects of purified macrophage RNases on granuloma fibroblasts with reference to silicosis. Acta Physiol Scand 1980 109 275–281 Received 10 Nov 1979 ISSN 0001-6772. Department of Medical Chemistry University of Turku, Finland

T alkaline RNases, designated RNase 1 and RNase ..., were isolated from the culture media of silica-treated and non-treated macrophages. The yield of RNase from the medium of silica-treated macrophages was 30% of that from the non-treated control The effects of these RNases on cultured granuloma fibroblasts and on granulation-tissue nuclei were studied. RNase 1 inhibited thymidine incorporation into fibroblasts except at low concentrations, where it was observed to be stimulatory RNase 1 also inhibited the protein synthesis of fibroblasts. The incorporation of cytidine into RNA in cultured fibroblasts was not affected by RNase 1 but the incorporation into isolated nuclei was decreased I pulse chase experiment RNase 1 increased the release of cytidine, but not that of thymidine from the cells RNase 2 had no effect on the protein or nucleic acid metabolism of the fibroblast or on the RNA metabolism of isolated nuclei, perhaps because of impermeability These experiment confirm that macrophage RNase activity is able to regulate the metabolism of granulation-tissue fibroblasts by increasing RNA degradation. Through this action it also regulates DNA and protein synthesis and other metabolic functions of those cells.

Key words RNA turnover DNA synthesis, protein synthesis, macrophage RNases, fibroblasts silica

... cells are stimulated to proliferate they first ...ulate RNA (Hanck & Green 1973 Johnson et ...76) The level of mRNA is a major rate-con...g factor in the ynthesis of those proteins, ... are necessary for mitosis and maintenance ...A synthesis. Changes in RNA turnover cause ... synthesis of proteins and finally also of ... (Abelson et al 1974 Johnson et al 1974)

...ica-treated macrophages release into the cul... medium a fibrogenic factor which increases ...ein synthesis in granulation-tissue fibroblasts ...pleston & Styles 1967 Aalto & Kulonen ...) We ha previously found that the culture ...um from rat peritoneal macrophages contains ...Nase activity which inhibit the synthesis of ...ins and DNA in fibroblasts (Aho & Kulonen ...)

...this paper we ha... tudied how the purified ...ses from the media of cultured ...ilica-treated ...non treated macrophages affect cultured granulation-tissue fibroblasts and nuclei isolated from experimental granulation tissue.

MATERIALS AND METHODS

Culture of macrophages

Rat peritoneal macrophages were harvested and washed as described by Aalto & Kulonen (1979). The washed cells were then transferred to culture flasks (Nunclon, N1475) (10^8 cells/174 cm^2 in 30 ml of serum-free Dulbecco' modification of Eagle Minimum Essential Medium, D-MEM buffered with HEPES and $NaHCO_3$ and containing 100 IU/ml penicillin G and 50 μg/ml streptomycin sulphat) and allowed to adhere for h. Nonadherent cells were ...emoved by rinsing and fresh medium added Where indicated the medium contained 0.15 mg/ml of SiO_2, Dörentrup quartz DQ L..., <5 μm The cultures were incubated at 37°C in an atmosphere of 95% air plus 5% CO_2 After 2 and 4 days the medium was changed, both non-treated and treated cultures received medium without silica. The media were collected and filtered through Millipore-filter (0.22 μm) to remov detached cells and SiO_2-particles

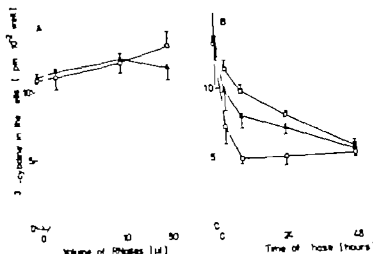


Fig. 2. The effect of RNase 1 on the incorporation (A) and release (B) of ^{3}H-cytidine in granulation-tissue fibroblasts. (A) Fresh culture medium with 2% FCS and RNase 1 as added to the confluent fibroblast cultures in microtest plates. After 4 h the cells ere detached and collected, and the radioactivity determined. (B) Confluent cell cultures were labeled for 4 h with ^{3}H-cytidine in a medium containing 5% FCS. Medium with no serum and 50 µl of enzyme solution was added to the cells. At the indicated times the cells were detached and collected and the radioactivity determined. No enzyme added (□—□). RNase 1 from 3–4 day medium of non-treated (0.45 units/ml) (○—○) and of silica-treated (0.15 units/ml) (△—△) macrophages. Averages ±S.E.M. (A 4, B = 6) are presented.

...rporation of radioactive CTP into ...d nuclei

...corporation of ^{3}H-CTP (TRK.339. The Radiochemical ...ntre, Amersham, U.K.), was measured by the ...d of Hooton & Hoffbrand (1977). The reaction ...t contained in total volume of 150 µl 17 µM ...f dGTP, dCTP and dATP, 7 mM $MgCl_2$, 0.1 M ... mM ATP, 0.7 mM EDTA, 1 mM β-mercapto...nol, 0.17 M Tris-HCl, pH 7.8, 0.2 µm ^{3}H-CTP ... corresponding to 75 µg DNA and 25 µl of the ...d RNases. The washed nuclei ere hydrolyzed in ...of 0.3 M KOH overnight at 37°C. The hydrolyzate ... precipitated by adding 0.3 ml of 1.04 M perchloric ...nd centrifuged at 1 000 g for 15 min. The supernatant ...collected into scintillation vial. The pellet was ...d once ...th 0.5 ml of 0. M perchloric acid, the ...ng combined ...th the first supernatant and the ...ctivity in the samples determined as described by ...en (19..).

...ULTS

...ation of RNase from macrophage media

...We separated two alkaline RNases from 60 ml ...re medium of each silica-treated and non-...ed macrophages. The olume of the final ...se solutions from the poly(G)-Sepharose 4B was adjusted to 6 ml. There were about equal amounts of RNase 1 and activities in the macrophage media (Fig. 1 A). The 0–2 day medium of non-treated macrophages gave the highest RNase activity (0.90 units/ml = 100 used as reference value) the 3–4 day medium from non-treated cells and the 0–2 day medium from silica-treated cells gave lower RNase activity (0.50 and 0.45 units/ml respectively) and the 3–4 day medium from the silica-treated cells gave the lowest RNase activity (0.15 units/ml) (Fig. 1 A).

Th effects of macrophage RNase on granulation-tissue fibroblast

RNase 2 had no effect on the thymidine incorporation into cultured granulation-tissue fibroblasts, but the incorporation was inhibited by RNase 1 (Fig. 1 B) depending on the amount of RNase activity in the medium. The low RNase 1 activity from the 3–4 day medium of silica-treated macrophages had no obvious effect but the higher RNase 1 activities from the three other media inhibited thymidine incorporation into granulation-tissue fibroblast by 50% (Fig. 1 B).

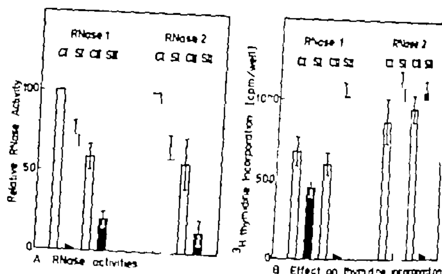


Fig 1 The relati e activities of RNases from macrophage media (A) and their eff cts on H thymidine incorporation into cultured granulation-tissue fibroblasts (B). RNases were purified from 60 ml of media from equal numbers of silica-treated and non-treated macrophages and the final volume of the RNase solutions was adjusted to 6 ml. The activities were determined with yeast RNA a substrate ^{3}H thymidine incorporation was determined in micro-te t plates in a total volume of 700 μl with 50 μl of RNase solution RNase purified from the 0– day medium of non-treated (C I) and of silica-treated (S I) macrophages. C II RNase purified from the 3–4 day medium of non-treated and S II of silica-treated macrophages Averages ±S E M (n=4) are presented

Purification of RNa e from macrophage media

The RNases were isolated as described in detail elsewhere (Aho submitted for publication) The macrophage medium was first dialyzed again t 0 05 M phosphate buffer pH 6 and then adsorbed onto an affinity column of protamine sulphate bound to Sepharose 4B (prepared according to Pharmacia instructions) The attached fraction was eluted with 1 M NaCl in phosphate buffer and desalted by dialysis against phosphate buffer The attached desalted fraction and the non-attached fraction were separately chromatographed on an affinity co lumn of poly(G) bound to Sepharose 4B and the material that attached were eluted with 1 M KCl and desalted by dialysis again t phosphate buffer The RNase attached to protamine ul phate was called RNase 1 and the non-attached one RNase 2

Enzymic activity of the purified RNases was determined with yeast RNA a substrate as described by Aho & Kulonen (1979) One unit of enzyme produced 1 mmol of acid soluble nucleotides per hour in these conditions

Production of experime t l gran lation ti ue

Granulation tissue was induced in adult rat of Wistar strain by viscose cellulose sponge (Viljanto & Kulonen 196_). The granulomas were collected after 14 days the surrounding capsules removed and the granulomas used immediately

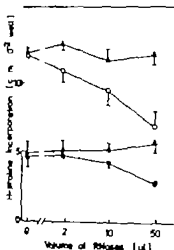
Culture of granulation-tissue fibrobla t

The sliced granulomas were treated with collagenase trypsin and hyaluronidase and the cell isolated a described by Ivaska (1973). Cells were cultured in growth medium as above but containing 10% foetal calf serum FCS (Flow Laboratories) When confluent, the cells were detached with 0.25% trypsin in Ca^{2+} Mg^{2+} free ... solution and then subcultured at a 1 4 dilution

The incorporation of ^{3}H-proline and ^{3}H-cytidine was studied with confluent cells and that of ^{3}H-thymidine with dividing cells at cell passages 4-8, using micro plates (Nunclon N1482/1-67008). The culture medium was changed and the sample added as indicated in the Figures After a 4 h incubation 0. μCi of L-(...) proline (TRK 3_3 The Radiochemical Centre Amersham U K) (5- H)-cytidine (TRK 195) or (methyl-^{3}H) thymidine (TRK 300) in 10 μl of medium was added and the incubation was continued for 24 h. The cells were detached with 0 25% trypsin collected onto glass fibre discs (GF/C Whatman) with an automatic cell harvester (made in our workshop) and the radioactivity in the ... determined a described by Runnemaa & Dobern (...

Is lation of ucle from e perimental granulation t s ue

The granulation tissue was homogenized in TEMMK (10 mM Tris HCl 1 mM EDTA 6 mM β-mercaptoethanol 4 mM MgCl$_2$ pH 7.8) with an Ultra-Turrax homogen zer (Janke & Kunkel Staufen i. Breisgau, Germany) using 1 g of tis ue per 5 ml of buffer as described earlie (Hershey et al 1973) The homogenate was filtered through a nylon cloth and the nuclei recovered by centrifugation at 800g for 5 min The pellet was washed ... time with TEM buffer (10 mM Tri HCl 1 mM EDTA ... mM β-mercaptoethanol pH 8 0) as described by Hershey et al (1973)



Effect of RNase 1 on ^{3}H-proline incorporation into ... of cultured granulation-tissue fibroblasts. Fresh ... medium without FCS and with RNase 1 was ... to confluent cell cultures. After 4 h incubation ... count of incorporated ^{3}H-proline in the cells and ... proteins was determined. The culture medium ... for 16 h with 6N PCA, the precipitated pro... collected on filter and the radioactivity determined. ... of RNase 1 from 3–4 day medium of non-treated ... (—●—●—) and of silica-treated (—△—△— ▲—▲—) ... phages on the incorporation of ^{3}H-proline into cells ... symbols) and into secreted proteins (closed symbols) ... S.E.M. (=4) are presented.

...-treated macrophages contains low RNase ac...

...ke of RNase into the cell

... differences in the molecular structures of ... se 1 and 2 are not yet clear. In SDS-acrylamide ... lectrophoresis they have the same molecular ... ht (Aho submitted for publication). The pres... experiments proved that only RNase 1 had ... pical effects on granulation-tissue fibroblasts ... on nuclei isolated from granulation tissue. The ... static effects of ribonuclease preparations have ... found to correlate well with the rate of uptake ... ribonuclease (Bartholeyns & Baudhuin 1976) ... eric RNase A was taken up into cell 10 to 15 ... faster than the monomeric RNase A. It is ... that an RNA component is attached to mac... age RNases (Aho submitted for publication) ... has a role in the uptake of RNase into the cell ... on not cytotoxic concentrations (Fig. 3) the ... eased DNA synthesis could result from this ... A component. In prokaryotes there is a primer

RNA which must be cleaved off by a very specific RNase before the elongation and maturation of RNA-attached DNA segments to chromosomal DNA take place (Sheinin et al 1978)

Effect of RNases on cell metabolism

The activity of alkaline RNase controls the amount of polyribosomes and protein synthesis in various tissues (reviews of Levy 1974 and Daoust & de Lamirande 1975). The macrophage RNases rapidly degrade polysomal and isolated rRNA to smaller fragments (Aho & Kulonen 1980). There is no turnover of the 18S and 28S ribosomal RNA in growing cultures (Kolodny 1975) and a great portion of the ribosomes is in use. The degradation of rRNA in growing fibroblast cultures by macrophage RNase could greatly decrease the cellular metabolism. However in confluent cells the degradation of rRNA does not play the primary role in the regulation of protein synthesis (Waldron et al 1977) because slow-growing cells have a high proportion of inactive ribosomes. In this case mRNA is a more susceptible target for RNase. The mRNA in the cytoplasm and nucleus is normally protected from RNases by proteins (Spirin 1972, Barrieux et al 1976) and a poly(A)-tail (Marbaix et al. 1975, Hieter et al. 1976) but it is susceptible to RNases during translation (Barnard 1969).

In this work the incorporation of cytidine and proline was studied in confluent cell cultures, which synthesize and secrete proteins but do not divide. In these cells the macrophage RNase decreased protein synthesis but the cellular metabolism was not decreased enough to be reflected as a decreased RNA synthesis during the 4 h incubation. The effect of RNase 1 on the nuclear RNA metabolism could be seen only after a 20 min incubation presumably because RNase 1 was not able to get through the nuclear membranes before. We suggest that RNase 2 could not get into the nuclei at all and, hence, had no effect on the cytidine incorporation.

The incorporation of thymidine was studied in rapidly growing granulation-tissue fibroblasts. In this case the effect of RNase 1 could be seen very clearly. With high amounts of RNase 1 the DNA synthesis was decreased as expected on the basis on earlier results. Low amounts of RNase increased thymidine incorporation into growing granulation-tissue fibroblasts. This may be due to the disturbance and destruction of some cellular components leading to reparative synthetic reactions, including

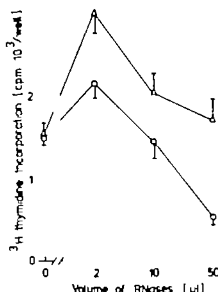


Fig 3 Effect of RNase 1 on the incorporation of H thymidine into cultured granulation-ti ue fibroblast Fresh culture medium with 5% FCS and RNase 1 wa added to growing fibroblast cultures After a 4 h incubation the amount of incorporated H thymidine in the cell was determined RNase 1 from 3–4 day medium of non treated (0 45 units/ml) (O—O) and of silica-treated (0 15 units/ml) (Δ—Δ) macrophages. Averages ±S E M (n=4) are presented

RNA synthesis of granulation-tissue fibroblasts was measured as H-cytidine incorporation into confluent cultures which no more synthesize DNA RNase 1 had no effect on the incorporation (Fig 2 A) In pulse-chase experiments RNase 1 increased greatly the release of H-cytidine from labeled RNA (Fig 2 B) It had no effect on the release of labeled thymidine from the granulation-tissue fibroblasts which were labeled during the growing phase (figures not presented)

The effect of RNase 1 on the incorporation of H thymidine was dependent on its concentra Small amounts of the enzyme (less than 4 rl 60 ng/well) stimulated the incorporation of thymidine but greater amounts of the enzyme w inhibitory (Fig 3) There was a linear corel between the log of the enzyme activity and the incorporation of thymidine

RNase 1 isolated from either the 0–2 day or 3–4 day medium of the silica-treated macrophages increased the incorporation of ^{3}H-cytidine into nuclear RNA about 50% when compared to the rate from an equal number of non-treated macrophages (Table 1) In contrast no clear differences could be seen in the effects of RNase 2 from different res The degradation of RNA could not be investig due to difficulties in separating rapidly CTP-labe RNA from DNA UTP does not incorporate at isolated nuclei of experimental granulation tis until destabilization of the nuclear membrane o curs

RNase 1 inhibited ^{3}H-proline incorporation i granulation tissue fibroblast and into the secre proteins The inhibition was dependent on RNase activity (Fig 4)

DISCUSSION

Effect of silica on the RNase activity

Silica crystals adsorb proteins on their surf from cell membranes causing membrane des tion (Kozin & McCarty 1976 Summerton c 1977) They also adsorb and immobilize RN which are liberated from subcellular partic peritoneal macrophages (Aho & Kulonen 1 From cultured macrophages silica rapidly lib and inactivates the RNases (Aho et al (19 a consequence the medium from 3–4 day cultu

Table 1 *Effects of RNases on the incorporation of CTP into nuclear RNA in experimental granulation tissue*

Nuclei were isolated from 14-day-old granulation tissue and incubated for 30 min with macrophage RNase presence of H-CTP as described in Material and Method Means ± S E M (n=4) are presented

Source of RNase	Incorporated radioactivity cpm/µg DNA RNase 1	RNase
Non treated macrophage medium 0– day	13 8± 6 4	15 0±9..
SiO$_2$-treated macrophage medium 0– days	21 5± 9	17 6±7 9
Non-treated macrophage medium 3–4 days	14 9± 8 8	20 8±7 1
SiO$_2$-treated macrophage medium 3–4 days	21 9±11 1	16 1±8 4

Effect of pretreatment with silica on RNase 1 from ( ) 0–2 day medium $P<0.05$ (b) 3 ium, $P<$

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DNA synthesis as discussed by Vasiliev et al (1970) In this work RNase I had no effect on the release of radioactivity from cells labeled with thymidine However Lehtinen (1979) has shown that RNase I has some nuclease activity also on DNA of nuclei isolated from granulation tissue

In conclusion the RNase activity in the macrophage culture medium varies with the age of the culture and is suppressed by silica treatment The RNase which is liberated from macrophages disturbs the metabolism of fibroblasts and this effect depends on the molecular structure and concentration

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cts of chronic nerve conduction block on formation euromuscular junctions and junctional ıE in the rat

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CANGIANO A., LØMO T LUTZEMBERGER, L. & SVEEN O Effects of chronic nerve conduction block on formation of neuromuscular junctions and junctional AChE in the rat. Acta Physiol Scand 1980 109 283–296 Received 12 Nov 1979 ISSN 0001 6772 Institute of Physiology University of Oslo Norway and Institute of Physiology University of Pisa, Italy

The development of ectopic n.m j s between the transplanted superficial fibular nerve and the soleus muscle has been studied in adult rats. Impulse conduction in the sciatic nerv as blocked chronically and synapse formation between the blocked fibular nerve and the paralysed soleus was compared with ynapse formation between non-blocked fibular nerves and denervated soleus muscles. Nerves with conduction block readily made new m j s. Thus 6 and 10–14 days after the onset of the block the number of newly innervated muscle fibres, the percentage of innervated fibres responding with action potentials and the frequency of m p p.s. at new junctions were comparable to that observed during innervation by non-blocked nerves. Muscle fibres innervated by both the original soleus nerve and the foreign fibular nerv were regularly encountered in the impulse blocked preparations. Junctions formed by impulse blocked fibular nerves had either no or ery little AChE activity 10–15 days after the onset of the block. The evidence for this was 1) weak staining for ChE 2) prolonged rise time and ½ decay time of m p p.s., 3) positive correlation between m p p. amplitude and ½ decay time and 4) insensitivity to anticholinesterases. In contrast, junctions formed by non-blocked fibular nerves had strong AChE activity by these criteria at corresponding times. AChE activity at the original soleus endplates was much reduced 10–15 days after the onset of conduction block.

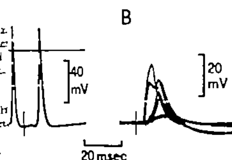
Key ords Neuromuscular junction formation, nerve conduction block impulse activity junctional AChE

ole of nerv impulse activity in the formation intenance of neuromuscular junctions has ly attracted considerable interest. Important anisms underlying the stabilization (Stent Changeux & Danchin 1976) elimination ien et al 1977 Thompson et al. 1979) and ution (Gordon et al 1976, Renaud et al. 1978) rve-muscle contact may all be influenced by se activity) On the other hand there is also nce that many events of synapse formation ccur in the absence of impulse activity (Harri-904 Kidokoro et al 1976)

terest has also focused on junctional acetyl-terase (AChE) activity which present evidence indicates is influenced both by evoked muscle activity (Giacobini et al 1973 Rubin et al 1978 Weinberg & Hall 1979 Lømo & Slater 1980 b) and by neural substances (Fernandez & Inestrosa 1976) whose release is stimulated by impulse activity (Younkin et al 1978)

The aim of the present work is two-fold First, to study synapse formation in vivo in the absence of impulse activity and second to see if in these circumstances, junctional AChE fails to appear as one would predict if impulse activity is of major importance

We have used a preparation in which trans-planted foreign nerve makes new ectopic ynapses



Multiaxonal innervation of single muscle fibres days of nerv block. A, action potentials evoked by ting first the tibial nerve and then the fibular nerve erent subthreshold endplat potentials evoked by ting the fibular nerve with stimuli of increasing h

Tracor Northern NS-570 digital signal averager he rising phase of each m p p to trigger the

second group of expts tape recorded m e.p p.s d into computer (NORD-1) which calculated the ng slues rise time ½ decay time and amplitude of and m p p s, mean and median values of these ters, usually based on 100 m p p.s from each m, amplitude histograms, frequency of m p p ally the Kendall coefficient of rank correlation m p p amplitude and ½ decay time or rise time ½ decay time of m p p from normal soleus fibres pproximately twice as long in the first as in the group of expts. This difference was most probably by much lower room temperature, perhaps as as 19°C, in the first group of expts. Unfortunately om temperature as not measured but must have much lower in the first group which was done in heated laboratory in November–December while cond group as done in very small laboratory the room temperature usually lies around 25–30°C ing of temperature is know to increase the average he ionic channel open time (Katz & Miledi 1972) he ½ decay time of m p p (Boyd & Martin 1956). t for differences in absolute values, the two groups ts gave similar result

ochemistry I nearly all expts, the distribution of as determined using the method of Buckley & on (1948) After physiological studies pairs of les th and ithout nerve block were then fixed ngle, dehydrated and cleared in benzyl benzoate.

ULTS

pse formation in th absence m impulse act n

pplication of a silicone rubber cuff around the lic nerve produced a clinically complete lysis in 19 animals. The paralysis developed ently about 1 day after the cuff had been applied and lasted until the acute experiment 6–15 days later

The sciatic nerve and the soleus muscle were then exposed and the nerve stimulated in situ. In all 19 animals stimulation below the cuff evoked strong contractions in the soleus The same stimulus applied above the cuff evoked no microscopically visible contraction in 14 animals (Fig. 1 A) and weak contractions in the remaining 5 In 8 animals with a complete conduction block in vivo impulse conduc tion partially recovered when the cuff was removed and the nerve muscle preparations placed in an oxygenated Ringers solution since stimulation above the cuff now caused weak contractions in the soleus. The reasons for this recovery are not clear but rapid return of nerve impulse conduction has been described in humans after sudden relief from chronic compression of the nerve (Hongell & Mattson 1971)

In each of the soleus muscles with a successful conduction block stimulation of the fibular nerve below the cuff evoked e p p.s or action potentials in surface fibres underlying the nerve growth (Table I) and a clear muscle contraction (Fig. 1 C) The new junctions were nearly always located several mm proximal to the old endplates where the transplanted fibular nerve had grown into the soleus. Stimulation of the tibial nerve evoked action potentials in many but not all surface fibres (Table I) and a contraction which was usually much larger than that evoked by stimulating the fibular nerve (Fig. 1 B) The tension produced by fibular nerve stimulation was usually much greater when this nerve stimulated alone (Fig. 1 C) than when it was superimposed on tibial nerve stimulation (Fig. 1 D) This indicates that most of the muscle fibres innervated by the fibular nerve were also innervated by the tibial nerve (Brown & Matthews 1960) That the fibular nerve stimulation sometimes added some tension to that produced by tibial nerve stimulation alone (Fig. 1 D) probably reflects the los of tibial nerve innervation on some muscle fibres through damage from the cuff (Table 1). The presence of double innervation was confirmed in many preparations by intracellular recordings from single muscle fibres (Fig. A) Sometimes stimulation of the fibular nerve evoked subthreshold e p p s and in uch cases a gradual increase in stimulation intensity frequently revealed several nitary e.p p (Fig. B) E idently two or more fibular nerve axons may innervate the same muscle fibre Sub-

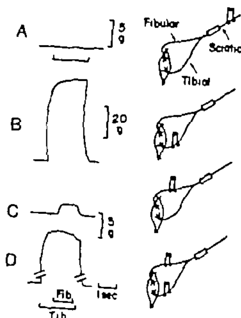


Fig 1 Isometric responses of a doubly innervated soleus muscle to nerve stimulation after 9 days of nerve block by a compressive cuff around the sciatic nerve Stimulation was *in vivo* at *100 Hz* for 1 or s A stimulation of sciatic nerve above the cuff B–D stimulation below the cuff of the tibial nerve alone in B the fibular nerve alone in C and of the tibial and the fibular nerve together in D

with a paralysed rat soleus muscle The paralysis is caused by a cuff around the sciatic nerve which blocks impulse activity both in the original nerve and in the foreign nerve This extends the work of Jansen et al (1973) who used a similar preparation but did not deal with the effects of the cuff on the foreign nerve By blocking the foreign nerve we could compare synapse formation by non-conduct ing and impulse-conducting nerves With the nota ble exception that very little AChE developed in the blocked preparation there were no gross differences between the two types of preparation In addition we have examined the effect of disuse on AChE activity at the original mature synapses and have extended and confirmed a previous report (Butler et al 1978) of a considerable reduction in AChE ac tivity

METHODS

Preparation of animal Male Wistar rats weighing 150–250 g were used Under ether or sodium pentobarbital anesthesia the superficial fibular nerve was cut distally in both legs and moved to the dorsal surface of the proximal half of the soleus muscles (for further details see Lømo & Slater 1978) After –3 weeks the animals were again anesthetized and an 8 mm long silicone rubber cuff (i.d

1 0–1 1 mm o d 4 mm) placed around the sciatic n on one side This caused complete paralysis of th within 1 day (see Cangiano et al 1977 for further de When this occurred the rats were anesthetized time and the contralateral soleus denervated by c the tibial nerve leaving the transplanted fibular ne tact The animals were then examined clinically for up to 15 days In most of the animals a c paralysis of the lower leg persisted on the cuffed side final control was performed at the time of the ac periment The sciatic nerve was stimulated a contractions in the soleus muscle looked for carefully the dissection microscope In most rats proximal st tion produced no visible response while distal stim elicited a vigorous muscle contraction These an retained for further study Evidently in these rats conduction had been successfully and perma blocked at the site of the cuff The remaining anim had incomplete conduction blocks at the time of the experiments usually also had shown clinical recovery from paralysis.

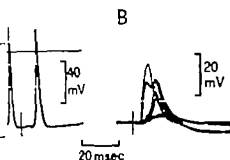
Contractile responses of the soleus to nerve stim proximally and distally to the cuff were also stu Isometric tension recordings (Fig 1). This was do vivo by connecting the Achilles tendon to a strain after the distal bellies of the medial and lateral gast mius muscles had been resected

Acute experiment The soleus muscles with the and the foreign nerve attached were removed from legs 6 to 15 days after onset of paralysis by cond block or section of the tibial nerve The muscles perfused in a chamber at room temperature with malian saline solution equilibrated with 95% O and CO (pH 7 3) The Ringer contained (mM) Na 5 Ca Mg 1 H PO 1 HCO 12 Cl 14 gluco

Miniature e p p s evoked e p p s and action po were recorded intracellularly with conventional pipettes filled with 4 M K-acetate or 3 M KCl at new (fibular nerve) and old (soleus nerve) junctions Old plates were localized by following the terminal fibres of the soleus nerve in the microscope and choo recording sites where the m e p p s were large and rising The new fibular synapses on the other hand lacked a precise morphological landmark and the recording site was not necessarily very close to the junctional area. Furthermore many muscle fibres had m and distributed synaptic contacts (Lømo & Slater 19 In any given fibre several penetrations (–30–90 μm were therefore made and records taken from sites a substantial fraction of the m.e p p.s were large and rising

In many experiments Neostigmine methyl (Roche) was added to the bathing fluid to obtain a concentration of 10^{-6} g/ml In other experiments the junctional ACh sensitivity was measured by ionophoresis of ACh and the sensitivity expressed as depolarization per nanocoulomb charge ejected from AChCl (3M) containing pipette

Computation of m e.p.p amplitude rise time decay time In an initial group of expts the decay time and amplitude of usually 100 m e p p at each junction were obtained by averaging the pow



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...second group of expts tape recorded m.e.p.p. ...al into computer (NORD-1) which calculated the ...ng values: rise time, ½ decay time and amplitude of ... m.e.p.p.s; mean and median values of these ...eters, usually based on 100 m.e.p.p.s from each ...n, amplitude histograms, frequency of m.e.p.p.s ...nally the Kendall's coefficient of rank correlation ... m.e.p.p. amplitude and ½ decay time or rise time ... ½ decay time of m.e.p.p.s from normal soleus fibres ...pproximately twice as long in the first as in the ... group of expts. This difference was most probably ...d by much lower room temperature, perhaps as ...s 10°C, in the first group of expt. Unfortunately ...om temperature was not measured but must have ...much lower in the first group which was done in ...heated laboratory in November–December while ...second group as done in very small laboratory ...re the room temperature usually lies around 25–30°C. ...ring of temperature is know to increase the average ...nic channel open time (Katz & Miledi 197?) ...e ½ decay time of m.e.p.p. (Boyd & Martin 1956). ...pt for differences in absolute values, the two groups ...ts gave similar results.

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In each of the soleus muscles with a successful conduction block stimulation of the fibular nerve below the cuff evoked e.p.p.s or action potentials in surface fibres underlying the nerve growth (Table I) and a clear muscle contraction (Fig. 1 C). The new junctions were nearly always located several mm proximal to the old endplates where the transplanted fibular nerve had grown into the soleus. Stimulation of the tibial nerve evoked action potentials in many but not all surface fibres (Table I) and contraction which was usually much larger than that evoked by stimulating the fibular nerve (Fig. 1 B). The tension produced by fibular nerve stimulation was usually much greater when this nerve stimulated alone (Fig. 1 C) than when it was superimposed on tibial nerve stimulation (Fig. 1 D). This indicates that most of the muscle fibres innervated by the fibular nerve were also innervated by the tibial nerve (Brown & Matthews 1960). That the fibular nerve stimulation sometimes added some tension to that produced by tibial nerve stimulation alone (Fig. 1 D) probably reflects the loss of tibial nerve innervation on some muscle fibres through damage from the cuff (Table 1). The presence of double innervation was confirmed in many preparations by intracellular recordings from single muscle fibres (Fig. A). Sometimes stimulation of the fibular nerve evoked subthreshold e.p.p.s and in such cases a gradual increase in stimulation intensity frequently revealed several unitary e.p.p.s (Fig. 2 B). Evidently two or more fibular nerve axons may innervate the same muscle fibre. Sub-

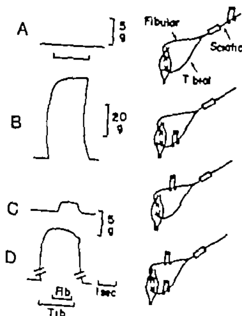


Fig 1 Isometric responses of a doubly innervated soleus muscle to nerve stimulation after 9 day of nerve block by a compressive *cuff around the sciatic nerve* Stimulation was in vivo at 100 Hz for 1 or 2 s A stimulation of sciatic nerve above the *cuff* B–D stimulation below the cuff of the tibial nerve alone in B the fibular nerve alone in C and of the *tibial and the fibular nerve together in D*

with a paralysed rat soleus muscle The paralysis is caused by a cuff around the sciatic nerve which blocks impulse activity both in the original nerve and in the foreign nerve This extends the work of Jansen et al (1973) who used a *similar preparation* but did not deal with the effects of the cuff on the foreign nerve By blocking the foreign nerve we could compare synapse formation by non-conducting and impulse-conducting nerves With the notable exception that very little AChE *developed in the* blocked preparation there were no gross differences between the two types of preparation In addition we have examined the effect of disuse on AChE activity at the original mature synapses and have extended and confirmed a previous report (Butler et al 1978) of a considerable reduction in AChE activity

METHODS

Preparation of animal Male Wistar rat weighing 150–250 g were used Under ether or sodium pentobarbital anesthesia the superficial fibular nerve was cut distally in both legs and moved to the dorsal surface of the proximal half of the soleus muscles (for further detail see Lømo & Slater 1978) After 2–3 weeks the animals were again anesthetized and an 8 mm long silicone rubber cuff (i.d 1 0–1 1 mm o d 4 mm) placed around the sciatic nerve on one side This caused complete paralysis of the [illegible] within 1 day (see Cangiano et al 1977 for further de[illegible] When this occurred the rats were anesthetized [illegible] time and the contralateral soleus denervated by [illegible] the tibial nerve leaving the transplanted fibular nerve [illegible]tact The animals were then examined clinically [illegible] for up to 15 days In most of the animals a [illegible] paralysis of the lower leg persisted on the cuffed side [illegible] final control was performed at the time of the [illegible]periment The sciatic nerve was stimulated [illegible] contractions in the soleus muscle looked for carefully [illegible] the dissection microscope In most rats proximal [illegible]tion produced no visible response while distal [illegible] elicited a vigorous muscle contraction These [illegible] retained for further study Evidently in these [illegible] conduction had been successfully and [illegible] blocked at the site of the cuff The remaining [illegible] had incomplete conduction blocks at the time of the [illegible] experiments usually also had shown clinical [illegible] recovery from paralysis.

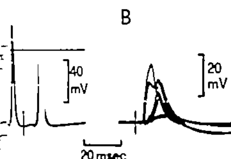
Contractile responses of the soleus to nerve [illegible] proximally and distally to the cuff were also [illegible] isometric tension recordings (Fig 1). This was [illegible] vivo by connecting the Achilles tendon to a [illegible] after the distal bellies of the medial and lateral [illegible]mius muscles had been resected

Acute experiment The soleus muscles [illegible] and the foreign nerve attached were removed from [illegible] legs 6 to 15 days after onset of paralysis by [illegible] block or section of the tibial nerve The muscles [illegible] perfused in a chamber at room temperature [illegible] malian saline solution equilibrated with 95% O [illegible] CO (pH 7 3) The Ringer contained (mM) Na [illegible] 5 Ca Mg 1 H PO 1 HCO 12 Cl 1[illegible]

Miniature e p p s evoked e p p s and action [illegible] were recorded intracellularly with conventional [illegible] pipettes filled with 4 M K-acetate or 3 m-KCl [illegible] (fibular nerve) and old (soleus nerve) junctions [illegible] plates were localized by following the terminal [illegible] of the soleus nerve in the microscope and [illegible] recording sites where the m e p p s were large [illegible] [illegible]ing. The new fibular synapses on the other [illegible] lacked a precise morphological landmark and the [illegible] recording site was not necessarily very close to the [illegible]tional area Furthermore many muscle fibres had [illegible] and distributed synaptic contacts (Lømo & Slater [illegible] In any given fibre several penetrations (250–500 μm [illegible] were therefore made and records taken from sites [illegible] a substantial fraction of the m e p p s were large [illegible] rising

In many experiments Neostigmine methylsulf[illegible] (Roche) was added to the bathing fluid to obtain [illegible] centration of 10^{-6} g/ml In other experiments the [illegible] junctional ACh sensitivity was measured by [illegible]phoresis of ACh and the sensitivity expressed [illegible] depolarization per nanocoulomb charge ejected [illegible] AChCl (3M) containing pipette

Computation of m e p p amplitude rise time and decay time In an initial group of expts the [illegible] decay time and amplitude of usually 100 m e p p s [illegible] each junction were o[illegible] a crapie [illegible]



Multiaxonal innervation of single muscle fibres days of nerve block. A, action potentials evoked by ting first the tibial nerve and then the fibular nerve. erent subthreshold endplate potentials evoked by ting the fibular nerv with stimuli of increasing h.

Tracor Northern NS-570 digital signal verager he rising phase of each m e p p. to trigger the

second group of expts tape recorded m.e.p p d into computer (NORD-1) hich calculated the ng values, rise time, ½ decay time and amplitude of led m p p s, mean and median values of these eters, usually based on 100 m p p from each m, amplitude histograms, frequency of m.e p p.s ally the Kendall coefficient of rank correlation m p p amplitude and ½ decay time or rise time ½ decay time of m p p from normal soleus fibres pproximately twice as long in the first as in the d group of xpts This difference was most probably by much lower room temperature, perhaps as 18°C, in the first group of expts. Unfortunately om temperature was not measured but must have much lower in the first group which was done in unheated laboratory in November–December while cond group as done in very small laboratory the room temperature usually lies around 25–30°C. ring of temperature is know to increase the average ate ionic channel open time (Katz & Miled 1972) he ½ decay time of m p p.s (Boyd & Martin 1956) rt for differences in absolute values, the two groups ps gave similar results.

histochemistry In nearly all xpts, the distribution of as determined using the method of Buckley & on (1968) After physiological studies pairs of les th and without nerve blocks were then fixed ample, dehydrated and cleared in benzyl benzoate

SULTS

apse formation in the abs nc rn impulse act ty

application of a silicone rubber cuff around the the nerve produced clinically complete alysis in 19 animals. The paralysis developed ately about 1 day after the cuff had been applied and lasted until the acute experiment 6–15 days later

The sciatic nerve and the soleus muscle were then exposed and the nerve stimulated in situ. In all 19 animals stimulation below the cuff evoked strong contractions in the soleus. The same stimulus applied above the cuff evoked no microscopically visible contraction in 14 animals (Fig. 1 A) and weak contractions in the remaining 5 In 8 animals with a complete conduction block in vivo impulse conduc tion partially recovered when the cuff was removed and the nerve muscle preparations placed in an oxygenated Ringers solution since stimulation above the cuff now caused weak contractions in the soleus The reasons for this recovery are not clear but rapid return of nerve impulse conduction has been described in humans after sudden relief from chronic compression of the nerve (Hongell & Mattson 1971)

In each of the soleus muscles with a successful conduction block stimulation of the fibular nerve below the cuff evoked e p.p.s or action potentials in surface fibres underlying the nerve growth (Table I) and a clear muscle contraction (Fig. 1 C) The new junctions were nearly always located several mm proximal to the old endplates where the transplanted fibular nerve had grown into the soleus Stimulation of the tibial nerve evoked action potentials in many but not all urface fibres (Table I) and contraction which was usually much larger than that evoked by stimulating the fibular nerve (Fig. 1 B) The tension produced by fibular nerve stimulation was usually much greater when this nerve timulated alone (Fig. 1 C) than when it wa superimposed on tibial nerve stimulation (Fig. 1 D) This indicates that most of the muscle fibres innervated by the fibular nerve were also innervated by the tibial nerve (Brown & Matthews 1960) That the fibular nerve stimulation sometimes added some tension to that produced by tibial nerve stimulation alone (Fig. 1 D) probably reflects the loss of tibial nerve innervation on some muscle fibres through damage from the cuff (Table 1) The presence of double innervation was confirmed in many preparations by intracellular recordings from single muscle fibres (Fig. A) Sometimes stimulation of the fibular nerve evoked subthreshold e.p p s and in such cases a gradual increase in stimulation intensity frequently revealed several nitary e p p s (Fig 2 B). E idently two or more fibular nerve axons may innervate the same muscle fibre Sub-

Table 1 *Surface fibres responding with e p.p s or action potentials to stimulation of fibular or soleus nerve* During fibular nerve stimulation only fibres underneath the growth of the fibular nerve were examined

	Days of paralysis	Innervated fibres (%)	Innervated fibres with AP (%)	Number of fibres	Number of muscles
Fibular nerve not blocked	6	7[illegible]	37	25	1
	10–14	89	69	89	3
Fibular nerve blocked	6	88	57	3[illegible]	1
	10–14	8[illegible]	72	159	5
Soleus nerve blocked	6	7[illegible]	7[illegible]	[illegible]5	1
	10–11	54	54	56	

threshold multiple e p p s were particularly frequent in the early stages of reinnervation The findings confirm those reported by Jansen et al (1973) and extend them by showing that the fibular nerve forms synapses even when the conduction block affects the fibular nerve itself

Many of the findings in the nerve impulse blocked soleus muscles were indistinguishable from those obtained when the soleus muscle was denervated and the transplanted fibular nerve left intact and allowed to conduct impulses In both types of preparation the extent of the foreign nerve growth was roughly similar and muscle fibres innervated by the fibular nerve were found with similar ease in both Thus after 6 and 10–14 days the blocked fibular nerve had innervated 88 % and 8[illegible] % respectively of the surface fibres lying underneath the nerve growth while the corresponding figures for the unblocked fibular nerve were 7[illegible] % and 89 % (Table 1)

There was no indication that the impulse blocked fibular nerve made unstable or transient synapses In muscles innervated by the intact fibular nerve the mean frequency of spontaneous m e p p s more than doubled between the 6th and the 10-14 (Table 2) During this period the endplates became more mature as shown by an increase in the [illegible] of evoked e.p p s that reaches threshold for [illegible] action potentials (Lomo & Slater 1978), by the ap[illegible] pearance of junctional AChE (Lomo & Slater [illegible] and below) and by the extent of their morpholog[illegible] maturation (Korneliussen & Sommerschild [illegible] Transmission at synapses made by the b[illegible] nerve was not obviously different from tra[illegible] sion at synapses made by the intact nerve [illegible] 10–14 days both the frequency of spontaneous m e p p s (Table 2) and the effectiveness of tra[illegible] mission as judged by the percentage of innerv[illegible] fibres responding with action potentials to stim[illegible] tion of the nerve were roughly similar in the [illegible] types of preparation (Table 1) The relatively [illegible] number of fibres in both preparations with [illegible] threshold e p p s may be attributed to several [illegible] tors such as 1) immature synapses releasing [illegible] than normal amounts of transmitter 2) low [illegible] membrane potentials resulting from the [illegible] paralysis and 3) effects of the cuff or the [illegible]

Table 2 *Characteristics of m e p p s including Kendall's coefficient of correlation (τ) between [illegible] and rise time at ectopic junctions formed by fibular nerve*

S E. of the mean indicated

	Days of paralysis	m e p p characteristics: No /sec	Rise time (msec)	Amplitude (mV)	τ amplitude vs rise time	Number of fibres	Number of muscles
Fibular nerve not blocked	6	0.41±0 1	3.03±0.21	0 39±0.037	0 [illegible]1±0.04	8	2
	10–14	1.02±0 14	1 14±0 03	0 36±0 015	0 02±0 0[illegible]	2[illegible]	2
Fibular nerve blocked	10–14	1.22±0 [illegible]0	1.80±0 05	0 44±0 024	0 06±0 03	[illegible]3	

3 *Surface fibres responding with 1 or more clearly disti guishable e p p.s to stimulation of fibular ... th stimuli of increasi g inte ity*

	Days of paralysis	Fibres innervated by 1 2, 3 or 4 axons (%) 1	2	3	4	Number of fibres	Number of muscles
...ner. not blocked	14	79	17	4		24	2
...ner. blocked	6	14	57	28		7	1
	10–14	58	30	10	2	50	3

...uction block on transmitter release. That the ... factors may be important was indicated by the ...rvation that after 10–14 days of conduction ... many e p p.s evoked by stimulation of the ...a nerve failed to elicit action potentials (Table ... hompson et al (1979) have observed a similar ... omenon in newborn rats where conduction ... the sciatic nerve had been chronically ...ed by plugs containing TTX which did not ... nerve degeneration. In these circumstances ... occurred striking reduction in indirect ...h tension attributable to generation of sub-...hold e.p p s

...neuronal innervation of single muscle fibres ...characteristic feature of ectopic synapse forma-... in this preparation (Kuffler et al. 1977 Lømo & ...r 1980*a*) Also in this respect synapses made ...locked and unblocked fibular nerves were simi-... As shown in Table 3 there was no qualitative ...rence between the two types of nerve fibres in ... capacity to multiply innervate single muscle ...s. There is suggestion of more extensive mul-...ronal innervation in the blocked preparation ... more data are required to establish if this is true

...ction of AChE ...new ...plates

...present study indicated striking differences in ...tional AChE activity at synapses made by im-...se blocked and non-blocked fibular nerves.

...AChE activity at neuromuscular junctions can be ...onstrated both histochemically and by the sen-...vity of endplate potentials to AChE inhibitors ...E inhibitors increase the amplitude and prolong ... time course of m.e.p p.s probably by allowing ...ased ACh to diffuse in the synaptic cleft and ...peatedly to activate ACh receptors (Katz & ...ledi 1973). This also results in a positive correla-... between amplitude and ½ decay time of ...e p p not normally present (Fig. 10 A) We have used these different indicators of junctional AChE activity to study its development at synapses made by either intact or impulse blocked fibular nerves

Histochemistry The ChE staining characteristics of denervated and nerve impulse blocked muscles were markedly different. 8 or more days after sec tion of the tibial nerve numerous intense and often well circumscribed plaques were present under neath the growth of impulse conducting fibular nerves (Pl 1 A) confirming previous results (Lømo & Slater 1980*b*) In contrast, there were only faint, elonged streaks of deposits in nerve impulse blocked muscles (pl. 1 C) The faint deposits in Pl. 1 C were typical of 9 nerve impulse blocked muscles all of which contracted to stimulation of the fibular nerve The deposits were present only in the region where the fibular nerve had made new synapses Their significance is uncertain since different kinds of ChE can be found along growing nerve terminals not making junctional synapses (Duchen 1970)

Time course of m.e.p.p.s 6 days after the onset of paralysis by denervation or nerve conduction block the duration of m e p p s was longer than normal in both types of preparation. Between 6 and 10 days the ½ decay time and the rise time decreased markedly in muscles innervated by impulse con-ducting fibular nerves (Fig. 3 Table 2) This de crease coincides in time with the histochemical ap-pearance of AChE activity (Lømo & Slater 1980*b*) In the nerve impulse blocked muscles on the other hand, the reduction in m.e.p.p. duration was much less pronounced After 10–14 days the ½ decay time was 71% (Fig. 4 A) and the rise time 58% (Table 2) longer than in the corresponding impulse conduct ing preparation The reduction which did occur may be attributed to (1) appearance of small amounts of AChE, (2) inactivation of adjacent synapses (Kuffler et al 1977 Lømo & Slater 1980) reducing the contribution of m.e.p.p s with a long time course due to electrotonic attenuation and (3) pos-

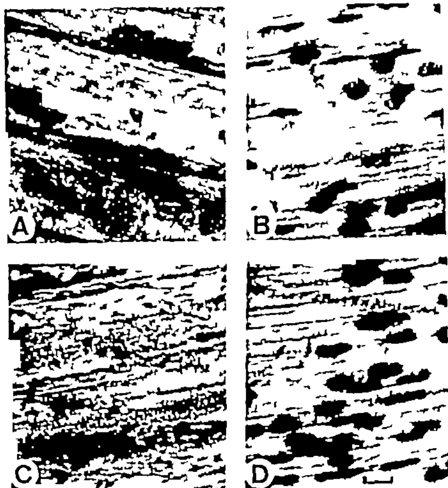
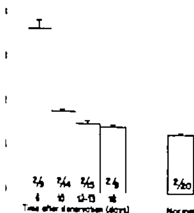


Plate 1 Representative deposits after staining for ChE (Buckley and Heaton 1968) in a soleus muscle denervated for 10 days (A and B) or paralysed for 10 days by a sciatic nerve block (C and D) A and C are from ectopic junctions formed by non-blocked and blocked transplanted fibular nerves respectively B and D from denervated and nerve blocked original soleus endplates respectively The two muscles are from the same rat and were stained together under identical condition

sible changes in the kinetics of the processes underlying transmission during synapse maturation (Sakmann & Brenner 1978)

M e p p s amplitude vs ½ decay time An increase in ½ decay time is no unequivocal sign of reduced AChE activity A similar increase would be caused by an increased specific membrane resistance (R_m) or capacitance (C_m) whereas changes in fibre diameter or internal resistance would have no such effect (see Gage 1976) Denervation causes R_m to increase (Westgaard 1975) and this may occur also after a chronic nerve impulse block which resembles denervation in many of its other effects (Lomo & Rosenthal 197? Cangiano et al 1977) An increased R_m however would not be expected to affect the correlation between m e p p amplitude and ½ decay time (or rise time) Normally there is no correlation between these parameters (Fig ... but if AChE is inhibited a positive correlation ... pears (Fig 10B) We have therefore examined ... correlation in impulse blocked muscles to ... evidence for a reduced AChE activity which ... dependent of changes in R_m

Fig 5 (upper part) shows scatter diagram ... m e p p amplitude against ½ decay time for ... resentative fibres Fig 5A is from a 6-day ... vated muscle and the observed positive correlation ($r=0.51$) is consistent with lack of AChE at ... time Common at this time is also the skew ... tude distribution and the low frequency of m e p ... (here 0 17 Hz) At 10 days a positive correlation ... no longer present in a denervated fibre ($r=0$ ... but still present in a nerve impulse blocked f... ($r=0.54$ C) Fig 4B su... the data treat...



Reduction in ½ decay time of m.e.p.p. at new fibular nerve junctions with increasing time after the soleus nerve. Bars give s of the mean. Column right refers to normal soleus junctions. Number of and fibres examined is indicated.

ty. After 10–14 days there is on average a ant positive correlation in nerve impulse d fibres ($r=0.37$, $P<0.02$) compared with ated fibres ($r=0.14$, $P>0.35$). The correlation might have been larger had all the m.e.p.p.s generated at one site. Instead some fibres had more separated inputs causing small, attenuated e.p.p.s to be recorded together with larger ones. This tends to give a negative correlation.

ts of Neostigmine. If it is true that the new es in nerve impulse blocked muscles fail to junctional AChE, then esterase inhibitors Neostigmine should have little or no effect p.p.s generated at these synapses. In denervated muscles, on the other hand, the m.e.p.p.s be prolonged. Fig. 6A, B shows that these ions are fulfilled. 13–15 days after the onset lysis the ½ decay time is relatively long in the impulse blocked muscles (Fig. 6B) and only slightly prolonged by the addition of of Neostigmine to the perfusion fluid. The gation was not statistically significant at the level. In denervated muscles, on the other the ½ decay time was initially relatively short Neostigmine prolonged it markedly (Fig. 6A). of these results were obtained by recording the same identified fibres before and after ation of Neostigmine. The experiments with gmine were done at a relatively low temperature (see Methods), and this probably accounts for relatively long duration of m.e.p.p.s in these experiments. Such an effect of temperature has been described previously (Boyd & Martin 1956).

Preparations with incomplete nerve impulse block. In a few rats the clinical paralysis began to wear off 10–15 days after the cuff had been applied. At this time the soleus muscle contracted weakly to stimulation of the sciatic nerve above the cuff indicating that some axons had recovered from the conduction block. These preparations were interesting because they offered and opportunity to study the time course of m.e.p.p.s in muscle fibres with varying degrees of previous activity. The level or duration of this activity is not known but an indication may be obtained by measuring the extrajunctional ACh sensitivity which is known to depend critically on the amount or type of previous muscle activity (Lømo & Westgaard 1975). Fig. 7 gives the result from such an incompletely blocked muscle. It is seen that the muscle fibres with the higher extrajunctional ACh sensitivity also had m.e.p.p.s with the longer rise times and ½ decay times. Furthermore, it was observed that fibres with high extrajunctional ACh sensitivity were thin and stained weakly for ChE at the end of the experiment while fibres with low extrajunctional ACh sensitivity were thick and stained intensely for ChE where the m.e.p.p.s had been recorded. If the longer m.e.p.p. rise times and ½ decay times reflect reduced junctional AChE activity, then the correlation shown in fig. 7 indicates that induction of junctional AChE and removal of extrajunctional ACh sensitivity are correlated phenomena, both

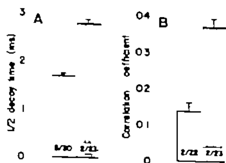


Fig. 4. ½ decay time of m.e.p.p. is longer (A) and Kendall correlation coefficient between ½ decay time and amplitude of m.e.p.p. more positive (B) at junctions formed by blocked fibular nerves (hatched columns) than by non-blocked fibular nerves (open columns) 10–14 days after onset of muscle paralysis. Bars give s.e. of the mean. Number of muscles and fibres examined is indicated.

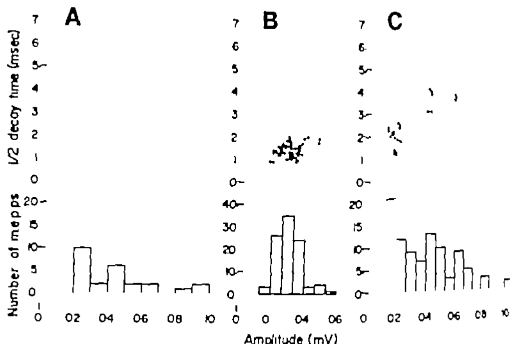


Fig 5 Scatter diagram of ½ decay times vs amplitude of individual m e p p s from a non-blocked fibular nerve junction (A) and 10 (B) days after cutting the soleus nerve and from a blocked fibular nerve junction 10 days after onset of paralysis (C upper row) Lower row shows distribution of m e p p amplitudes from the same 3 junctions

probably depending on muscle activity in a graded manner

Junctional AChE at original nerve impulse blocked synapses In these preparations impulse conduction was blocked not only in the foreign nerve but also in the original nerve to the soleus muscle It was therefore possible to examine how lack of impulse activity affected the maintenance of AChE at well established mature synapses

Histochemistry Histochemical staining for ChE did not give any clear indication of an effect of the nerve block Both nerve impulse blocked and denervated original synapses stained well for ChE (Fig 1 B D) and neither differed notably from the staining of normal synapses We therefore looked for more subtle changes by examining the m e p p s of the nerve impulse blocked synapses along the lines described above for the new synapses

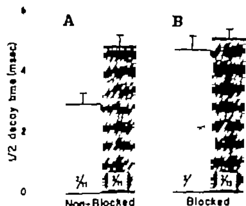


Fig 6 Effect of Neostigmine on ½ decay time of m e p p at ectopic junctions formed by non-blocked (A) and blocked (B) fibular nerves 13–15 days after onset of muscle paralysis. Open columns before and hatched columns after addition of 10^{-6} g/ml of Neostigmine to the perfusion fluid Bars give S E. of the mean Number of muscles and of fibres is indicated

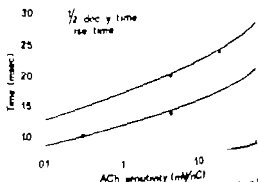


Fig 7 Positive correlation between m e p p ½ decay time (●) or rise time (O) and extrajunctional ACh sensitivity of the fibres from which the m e p p s were obtained m e p p were generated at junctions formed by the fibular axons in a preparation where some of the ... had already recovered from the block starting 17 ... earlier Kendall's correlation coefficients were 0 ... (decay time) and 0 83 (rise t...



Fig. 8. ½ decay times of m.e.p.p.s at original soleus endplates in normal muscles (A and B, open columns), after 10–14 days of conduction block in the tibial nerve (A, hatched columns) and after addition of 10⁻ g/ml Neostigmine to the perfusion fluid (B, hatched columns). Bars are S.E. of the mean. Number of muscles and of fibres is indicated.

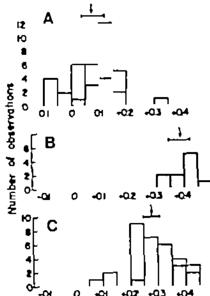


Fig. 9. Distribution of Kendall's coefficient of correlation between ½ decay time and amplitude of m.e.p.p.s at original soleus endplates in normal muscles (A), in normal muscle exposed to 10⁻ g/ml of Neostigmine (B) and in muscles paralysed for 10–12 days by chronic sciatic nerve block (C). Hatched columns in A and B represent subpopulation of normal fibres before (A) and after (B) application of Neostigmine and in C fibres from the longest paralysed muscle (14 days). Arrows give median values and horizontal bars 95% confidence limits.

Time courses of m.e.p.p.s. The chronic nerve conduction block caused a significant increase in the duration of the m.e.p.p.s at the original endplates. On average the rise time became approximately ... longer (Table 4) and the ½ decay time approximately 100% (Fig. 8A) longer than normal after ... days of conduction block. Similar but less pronounced increases occurred at normal soleus endplates after Neostigmine (10⁻ g/ml) had been added to the perfusion fluid (Fig. 8B).

M.e.p.p. amplitude vs ½ decay times. Chronic nerve conduction block of 10–14 days duration caused the appearance of a positive correlation between m.e.p.p. ½ decay time and amplitude. The median coefficient of correlation (τ) was 0.28 and the correlation was significantly positive at the 1% level for 90% of the fibres (Fig. 9C). In normal muscles, on the other hand, the median τ was only ... (Fig. 9A) and only 6% of the fibres showed significant correlation at the 1% level. The positive correlation induced by the block may be compared with that induced in normal fibres by Neostigmine (Fig. 9A and B, hatched columns). After Neostigmine the median τ increased from 0.08 to 0.36. This is nearly the same as the value 0.35 obtained in the preparation with the longest conduction block (14 days, Fig. 9C, hatched columns). Fig. 10A shows an example of the lack of correlation in a normal fibre ($r = 0.04$) and the positive correlation ($r = 0.40$)

Table 4. *Characteristics of m.e.p.p.s including Kendall's coefficient of correlation* (τ) *between amplitude and rise time at original soleus endplates.* ... of the mean indicated

	m.e.p.p. characteristics					
	No./sec	Rise time msec	Amplitude mV	τ ampl. vs. rise time	Number of fibres	Number of muscles
Normal soleus endplates	1.38±0.1	0.89±0.02	0.38±0.01	0.08±0.0_	36	4
Nerve fibers blocked 10–14 days	_.20±0.14	1.43±0.04	0.60±0.03	0.16±0.02	31	4

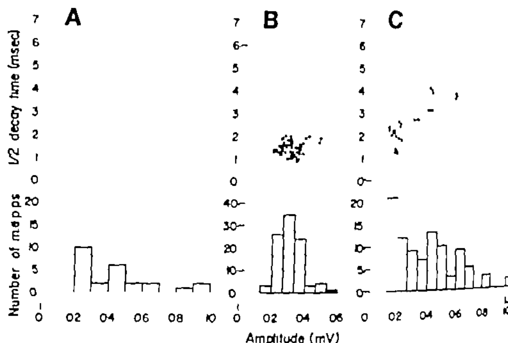


Fig 5 Scatter diagram of ½ decay times vs amplitude of individual m e p p s from a non-blocked fibular nerve junction (A) and 10 (B) days after cutting the soleus nerve and from a blocked fibular nerve junction 10 days after onset of e paralysis (C upper row) Lower row shows distribution of m e p p amplitudes from the same 3 junctions

probably depending on muscle activity in a graded manner

Junctional AChE at original nerve impulse blocked synapses In these preparations impulse conduction was blocked not only in the foreign nerve but also in the original nerve to the soleus muscle It was therefore possible to examine how lack of impulse activity affected the maintenance of AChE at well established mature synapses

Histochemistry Histochemical staining for Ch did not give any clear indication of an effect of the nerve block Both nerve impulse blocked and de nervated original synapses stained well for ChE (Fig 1 B D) and neither differed notably from the sta ing of normal synapses We therefore looked f more subtle changes by examining the m e p p the nerve impulse blocked synapses along the li described above for the new synapses.

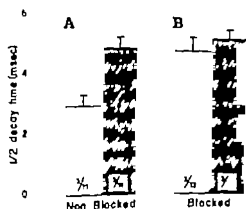


Fig 6 Effect of Neostigmine on ½ decay time of m e p p s at ectopic junctions formed by non-blocked (A) and blocked (B) fibular nerves 13–15 days after onset of muscle paralysis. Open columns before and hatched columns after addition of 10^{-6} g/ml of Neostigmine to the perfusion fluid Bars give S.E. of the mean Number of muscles and of fibres is indicated

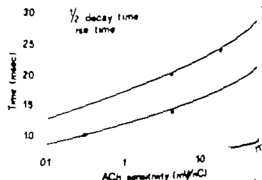


Fig 7 Positive correlation between m e p p ½ decay (●) or rise time (O) and extrajunctional ACh sensitiv the fibres from which the m e p p s were obtained T m e p p s were generated at junctions formed by fo fibular axons in a preparation where some of the had already recovered from the block starting 1 earlier Kendall's correlation coefficients ere 0 (cay time) and 0 83 (rise

e 5 *Amplitude of m.e p p at original soleus junctions ("old") a d ectopic junctions formed by fibular ("new") before nd after exposure to 10⁻⁴ g/ml of Neostigmi e*

of the time indicated

	m p p amplitude mV			
	Before Neostigmine	After Neostigmine	(%) increase	Number of fibres
endplates				
leus nerve not blocked	0 41±0 14	0 61±0_4	49	9
leus nerve blocked 1–15 days	0.50±0 13	0 53±0 14	6	15
endplates				
bular nerve not blocked	0.34±0 07	0 40±0 10	18	11
bular nerve blocked 1–15 days	0 49±0.14	0 44±0.14	−10	13

pse formation in the absence n impulse ctivity

cuff around the sciatic nerve blocked impulse ity both in the original nerve to the soleus and he transplanted fibular nerve The first block necessary to paralyse the soleus and render it ptive to innervation by the fibular nerve (Jan- et al. 1973) A similar effect could have been ved by cutting the original nerve to denervate muscle (Fex & Thesleff 1967) The second k prevented nerve impulses from being con- ed into the distal 1 2 cm of the transplanted r fibres and allowed us to ask if this would ct the ability of the nerve to form synapses The ing finding was that blocked nerves innervated roximately the same percentages of muscle s as unblocked nerves did in denervated mus- Furthermore the synapses developed in much same way in the two preparations cept that little junctional AChE appeared in the blocked aration (see below) This shows that neither e impulse activity evoked transmitter release evoked muscle activity is essential for the for ion of functional neuromuscular connections s extends to a mammalian in vivo preparation vious reports of synapse formation in the pre ce of impulse blocking agents in tadpoles (Harri- 1904) and in vitro between explants of rat pinal d and L6—myotubes (Kidokoro et al. 1976) no & Slater (1980) reported that high and sta- ACh sensitivity develops underneath surgically ated foreign nerve terminals indicating that the uction of this post junctional specialization does t require impulse activity The present work is nsistent with this and indicates in addition that presynaptic structures for transmitter release also form in the absence of impulse activity

While the essential events in synapse formation clearly occur in the absence of impulse activity our tests do not exclude more subtle effects of impulse activity on for example efficiency of transmission or on number and distribution of new synapses on single fibres

Cond ction block by erve uffs

Criticism has been raised against the use of cuffs for blocking nerve impulse conduction (Blunt & Vrbová, 1975) The mechanism behind the block is not clear and the cuffs may have different unknown effects.

In these experiments we used plain silicone cuffs contain- ing no local anaesthetics or other drugs. The block de- veloped rapidly about 20–30 h after application of the cuff in contrast to the immediate block caused by cuff con- taining local anaesthetics. The internal diameter of the cuff was critical for successful block and variations of 1 2 tenths of mm made the block either ineffective or caused extensive nerve degeneration It is known that compression may lead to local demyelination (Ochoa et al. 1971) which by itself blocks impulse conduction (Ras- minsky & Sears 1972). It seems likely that the cuff blocked impulse conduction by compressing the nerv

The delayed onset of the conduction block may be attributed to reactive edema within the cuff which could gradually increase the compression. The fact that injec tion of cortisone prevents the block provides evidence for such mechanism (Cangiano & Lutzemberger unpub- lished). It is interesting that the immediate block caused by cuffs containing local anaesthetic is sometimes tran- sient and that second longlasting block develops about 20–30 hours after application of the cuff (Cangiano et al. 1977) Also with cuffs containing anaesthetics the internal diameter is critical It is likely that these cuffs block im- pulse conduction by causing an immediate but transient

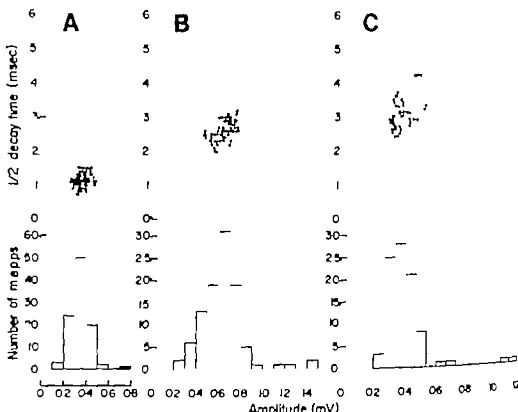


Fig 10 Scatter diagram of ½ decay times vs amplitude of individual m.e.p.p.s from a normal soleus nerve endplate before (A) and after (B) exposure to 10⁻ g/ml of Neostigmine and from a soleus nerve endplate after a sciatic anesthesia of 12 days duration (C) Lower row shows m.e.p.p. amplitude distributions from the same 3 endplates.

obtained in the same fibre 60 min after application of Neostigmine Fig 10C is from a fibre after 12 days of nerve conduction block There is a positive correlation (r=0 36) resembling that found in Neostigmine treated normal fibres (Fig 9B)

Effect of Neostigmine Experiments with Neostigmine produced perhaps the clearest evidence

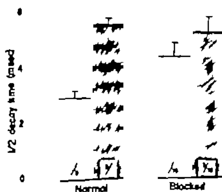


Fig 11 ½ decay times of m.e.p.p.s at original soleus endplates before (open columns) and after (hatched columns) application of 10^{-6} g/ml of Neostigmine in normal muscles and in muscles paralysed for 13–15 days by a chronic sciatic nerve block Bars give S.E. of the mean Number of muscles and of fibres is indicated

that the conduction block reduces AChE activity at mature endplates In the impulse blocked preparation Neostigmine increased only moderately (21%) the ½ decay time of m.e.p.p.s already abnormally long as a result of the paralysis (Fig. 11B). In normal preparations on the other hand, the increase was much larger (86% Fig 11A) It is possible that these differences would have been even larger had these experiments with Neostigmine been done at the same high room temperature as the previous group of expts (see Methods) A low temperature is known to prolong m.e.p.p. time course and to reduce the prolonging effect of esterase inhibitors (Boyd & Martin 1956)

DISCUSSION

This work deals in particular with two aspects of neuromuscular synapse formation (1) the ability of nerves to form synapses in the absence of impulses conducted to the nerve terminals and evoked in the muscle and (2) the induction of AChE at newly formed endplates and the maintenance of AChE at established mature synapses

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block due to the local anaesthetic and a later prolonged block due to compression

Whatever the exact mechanism behind the block it is clear that blocked nerve fibres do not differ in any fundamental way from intact nerve fibres with respect to conduction of impulses distal to the block or capacity to form new functional synapses The competence of the nerve in these respects argues against serious injurious effects of the cuffs used in the present experiments

Impaired formation of AChE at new synapses

The nerve conduction block prevented the appearance of AChE at the developing new synapses almost completely This result is consistent with other evidence that formation of junctional AChE requires evoked muscle activity (Lomo & Slater 1980*b*) By themselves however the present experiments do not distinguish between neural and muscular controlling factors because the cuffs may not only interfere with muscle activity but possibly also with axonal transport of neurotrophic substances controlling AChE formation (Fernandes & Inestrosa 1976 Younkin et al 1978) and with the transport and release of AChE itself (Skau & Brimijoin 1978) The low AChE activity present at some impulse blocked synapses may be taken as evidence of an impulse independent control mechanism However this marginal activity may also be accounted for by fibrillatory activity known to develop in nerve blocked rat muscles (Lomo & Rosenthal 1972) Fibrillatory activity has been shown to lower slightly denervation hypersensitivity (Purves & Sakmann 1974) The same muscle activity could by virtue of its reciprocal effects on extrajunctional ACh sensitivity and junctional AChE activity (Lomo & Slater 1980*b*) lead to the formation of small amounts of junctional AChE Finally the possibility cannot be excluded that some undetected impulse activity may arise in these preparations from temporarily failing conduction blocks or impulse generation near the site of compression (Howe et al 1978)

Reduction in AChE at mature endplates

Nerve impulse activity was shown to be important not only for the formation of AChE at new synapses but also for its maintenance at old synapses The electrophysiological evidence indicated a major reduction in AChE acting on nerve released ACh ("functional AChE") at impulse blocked endplates The sensitivity to Neostigmine was markedly reduced and in a few fibres completely absent.

The major reduction in "functional AChE" is in contrast to the considerable histochemically detectable ChE activity at both denervated and impulse blocked endplates This raises the possibility that some of the endplate Cholinesterases may not normally act on nerve released ACh. Such a possibility may not seem unreasonable considering the different location of Ch esterases relative to ACh receptors (Fertuck & Salpeter 1976) the very long persistence after denervation of some forms of ChE relative to the rapid disappearance of the 16S form found mainly at endplates (Hall 1973 Vigny et al 1976) and finally the reported persistance after denervation of histochemically detectable ChE activity not affecting focally applied ACh (Pecot Dechavassine 1968)

The reduction in "functional AChE" shown here is consistent with results recently obtained by [illegible]ler et al (1978) These authors assayed total ChE of endplate regions biochemically and showed that chronic nerve impulse block by tetrodotoxin had the same effect as denervation on ChE activity in soleus muscle endplates

In conclusion these and previous results (Lomo & Slater 1978 and 1980*a* and *b*) suggest the following roles of muscle activity in synapse formation In the absence of activity muscle becomes innervable New synapses may then form independently of nerve and muscle activity The new innervation reestablishes muscle activity which causes extrajunctional ACh sensitivity to disappear junctional AChE to appear and the muscle to become refractory to further innervation.

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...n-autonomic component in bradycardia ...endurance trained men at rest and during exercise

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LEWIS S F NYLANDER, E. GAD P & ARESKOG N H. Non-autonomic component in bradycardia of endurance trained men at rest and during exercise. Acta Physiol Scand 1980 109 297–305 Received 13 Nov 1979 ISSN 0001-6772. August Krogh Institute University of Copenhagen, Denmark and Department of Clinical Physiology Regionsjukhuset, Linköping University S eden

Autonomic nervous alterations have generally been held responsible for the bradycardia of the endurance athlete In order to determine whether there is also non-autonomic component in the bradycardia of long-term training we compared the intrinsic heart rate (HR) of highly trained bicyclists (heart olume: 995±155 ml) with that of untrained men (heart volume: 805±195 ml) at rest and during bicycle ergometer ercise at 50 75 and 100% of maximal oxygen uptake (V max) Intrinsic HR was achieved by combined vagal and beta-adrenergic blockade with atropine and propranolol or metoprolol (cardioselective) injected intravenously Intrinsic HR was significantly lower in trained (T) than in untrained (UT) at rest and at all levels of xercise. The chronotropic reserve from resting HR to maximal HR was identical in the two groups Nearly identical intrinsic HRs were achieved with atropine and either beta-adrenergic antagonist. HR differences between T and UT were very similar in magnitude—approximately 13 beats/min—at rest and during xercise at given percentage of V max, with and without autonomic blockade. Evidence is thus provided for non-autonomic component in the bradycardia of well-trained men which may be responsible for parallel downward shift in the relationship between HR and percentage of V_{O_2} max. The lower intrinsic HR in well-trained men might be explained by i.a. the cardiac enlargement

Key ords Physical training, bradycardia, autonomic nervous blockade propranolol, metoprolol

...ugh one of the hallmarks of the endurance ...te is a slower heart rate (HR) at rest and during ...se the mechanisms involved are still con...rsial (Scheuer & Tipton 1977). Most studies ...focused on the role of the autonomic nervous ...m in the training bradycardia (Brundin & Cer...ro 1975 Ekblom et al 1973 Winder et al. ...) However it also has been proposed that a ...utonomic component i e a lower intrinsic ...ay be involved (Badeer 1975 Sutton et al ...). Animal studies employing wide variety of ...ds provide findings in favour of (Barnard et al ... Bolter et al 1973 Hughson et al. 1977 Sig...son et al. 1977) and opposed to (Penparkgul & ...uer 1970 Tipton et al. 1977) a lower intrinsic ...after training. In man combined parasympathetic and beta-adrenergic blockade with atropine and propranolol has been used to study intrinsic HR at rest and during exercise (Jose & Collison 1970, Jose et al 1970 Nordenfeldt 1971 Sutton et al. 1967) We recently demonstrated reduced intrinsic HR at a given submaximal workload, but not at rest or maximal exercise after *short term* endurance training in man (Lewis et al 1980)

The purpose of the present study was to examine the non-autonomic component of the bradycardia at rest and during submaximal and maximal xercise after very intense *long-term* endurance training.

This study was performed while Steven Lewis was George C. Marshall Fellow His present address is. Di ...sion of Cardiology University of Texas, Southwestern Medical School, Dallas, Texas, 75235 USA.

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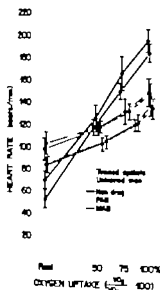
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[Fig. 1.] Heart rate in trained and untrained at rest and [during] exercise requiring approximately 50, 75 and 100% [of maxi]mal oxygen uptake without treatment and with [autono]mic blockade. Means ±1 S D PAB=Propranolol + [atropi]ne blockade MAB Metoprolol+atropine-block

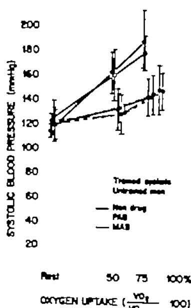


Fig. 2. Systolic blood pressure at rest and during submaximal exercise in trained and untrained without treatment and with autonomic blockade. Mean ± 1 S D Abbreviations as in Fig. 1

[... f]or repeated measures were performed to determine [st]atistical significance of observed differences among [th]e treatments within each group. If significant [F] was obtained differences between each pair of [trea]tment means were compared with Duncan New Multiple Range Test (Snedecor & Cochran). For all tests [the] null was accepted as significant if $P<0.05$ Correlation coefficients were calculated by the method of least [square]s.

[RES]ULTS

[Hear]t volume

[The a]thletes had significantly larger heart volumes [than] the untrained men, 995 ml vs 805 ml ($P<0.05$) [(Tab]le 1). The heart volumes per m² body surface [and] per kg body weight were also significantly [large]r in the trained than in the untrained group 540 [vs 4]39 ml/m² ($P<0.05$) and 15.0 vs 11.5 ml/kg [($P<0.0$]01), respectively. In the trained and untrained [grou]ps combined ($n=16$) there was a negative cor[relat]ion between the heart volume in ml/m² BSA [and] the resting HR without blockade ($R=-0.64$) [and] after PAB and MAB ($R=-0.41$ and -0.43 re[spect]ively)

[Hear]t rate

[With]out drugs HR was lower in the T vs. the UT [grou]p at rest and at all levels of exercise up to maximal (Fig. 1) HR was 53 vs. 70 beats/min ($P<0.05$) at rest, 118 vs 126 beats/min at 50% V max 150 vs. 166 beats/min at 75% V max ($P<0.05$) and 184 vs. 195 beats/min at V max ($P<0.05$) in the T and UT groups respectively

After combined parasympathetic and beta adrenergic blockade with atropine and propranolol HR was also lower in the T vs. the UT group at rest and at all levels of exercise up to maximum (Fig. 1) HR was 84 vs. 103 beats/min at rest, 105 vs. 118 beats/min at 50% V max, 122, s 132 beats/min at 75% V_{O_2} max and 135 vs 148 beats/min at V max, in the T and UT groups, respectively ($P<0.05$ for all comparisons) Very similar HR results were obtained after combined autonomic blockade with atropine and metoprolol (Fig. 1) Maximal HR was 46 and 49 beats/min lower after autonomic blockade with each beta-adrenergic antagonist in the T and UT groups respectively (Fig. 1)

Systolic blood pressure

Systolic blood pressure was lower in the trained than in the untrained men at rest (114 vs. 125 mmHg, respectively $P<0.05$) However this difference was not present after autonomic blockade

Table 1 *Physical characteristics of subjects*
Mean ± 1 S D

Group	Age (yr)	Height (cm)	Weight (kg)	Heart volume (ml)	V max (mmol kg min⁻¹)
Sedentary men (n = 8)	26±4	181±11	70±8	805±195	..0±0 3
Elite cyclists (n = 8)	21±2	177± 4	67±5	995±155	3 4±0..

This was accomplished by comparing the intrinsic HR of highly trained competitive bicyclists with that of untrained men Parasympathetic blockade was achieved with atropine and beta adrenergic blockade with propranolol a non-cardioselective antagonist or metoprolol a cardioselective antagonist in order to determine whether the results of previous intrinsic HR studies may have been affected by the exclusive use of propranolol as beta-blocker

METHODS

Subjects

8 elite long-distance bicyclists (trained = T) and 8 sedentary men (untrained = UT) volunteered as participants in this study All the elite cyclists had been bicycling 450–700 km/week for 5–11 years prior to testing and had been cycling competitively for at least 5 years The sedentary men were students or laboratory personnel who had never been competitive endurance athletes and who had not participated in regular training for at least one year immediately prior to the study Physical characteristics of the subjects are given in Table 1

Procedures

After a day of preliminary submaximal and maximal exercise tests all subjects were tested on 3 separate days under the following conditions. (1) without drugs ( ) after i v injection of atropine sulfate plus propranolol hydrochloride and (3) after i v injection of atropine sulfate plus metoprolol tartrate Test days were at least one week apart in the sedentary group and in the cyclists no tests were administered for at least 5 days after one of the drug treatments The conditions were administered in a random order Each subject was tested at approximately the same time each day The subjects fasted and abstained from smoking for at least 2 h prior to arriving at the laboratory and did not perform any strenuous exercise prior to being tested each day

On each day resting HR was determined after at least 20 min rest in the supine position On drug days the subjects remained supine and drug infusions were made through a catheter placed in an antecubital vein First 0 04 mg/kg body weight atropine and then either 0..5 mg/kg propranolol a non-selective beta-receptor antagonist or 0.5 mg/kg metoprolol a cardio-selecti e betablocker without intrinsic activity (Ablad et al 1975) were injected ... total time of 4–5 min The subject remained supine ... min after completion of the propranolol or metoprolol injection prior to measurement of the resting ... None of the subjects complained of anticholinergic ... effects of the atropine during the tests.

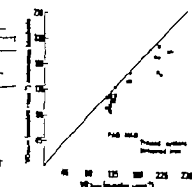
On each day the subjects then performed 3 exercise tests on a bicycle ergometer in the upright position ... first test was performed for 6 min at a ... approximately 50 % of maximal oxygen uptake (V_{O_2} ... as determined by preliminary testing After a 15 min ... second exercise test was performed for 6 min ... ty approximately equal to 75 % V_{O_2} max. In the ... experiments the subjects were given an additional ... 0 05 mg/kg propranolol or 0 1 mg/kg metoprolol ... to the second submaximal test Following the second ... maximal test the subjects rested for 10 min V_{O_2} max ... then determined with stepwise increased ... exhausted the subjects within 4–6 min

Expired air was collected in Douglas bags during ... last 2 min of submaximal exercise and the last ... maximal work HR was recorded continuously ... exercise Fingertip blood for determinations of lactate ... concentration was obtained at rest, min after each ... maximal exercise and and 5 m n after maximal exercise ... Systolic blood pressure was obtained by the cuff ... at rest on the bicycle ergometer immediately before ... ginning each submaximal test and during the fifth ... of submaximal work

The sedentary group was tested on a Krogh ... ergometer and the elite cyclists were tested on an E... bicycle ergometer (type EM369) Expired air volume ... measured with a balanced spirometer or ... centages of O and CO in expired air were determined ... the Scholander method or by a Servomex O_2 analyzer ... Beckman model LB- CO_2 analyzer which were ... regularly by the Scholander method Oxygen uptake ... calculated according to standard procedures HR ... determined from ECG tracings Blood lactate con... tions were determined by fluorometric and spectrophotometric methods (Jorfeldt & Juhlin-Dannfelt 197..., ...dahn 1959)

Che t X rays were obtained in the standing ... with a film-focal distance of 1.5 m The heart ... were determined by one radiologist according to ... (1939) The relative heart volume was calculated by ... lation of the total heart volume to the body surface ... according to DuBois formula.

Between the groups statistical comparisons were ... by t-tests for non-paired ... wo way analyses ...



Maximal oxygen uptake (V_{O_2} max) achieved [illegible] autonomic blockade compared to V_{O_2} max in the [illegible] state in trained and untrained men. (45 [illegible] l/min.)

[illegible] blood lactate was lower ($P<0.05$) after au[tonom]ic blockade with each beta-blocker than [u]n[der n]ormal conditions (when however the per[forme]d work load was considerably higher). In the [UT gro]up blood lactate at 75% V max and after [maxim]al exercise was higher ($P<0.05$) after meto[prolo]l and atropine than after propranolol and at[ropin]e.

[DISC]USSION

[The] cyclists exhibited several of the well-[know]n characteristics of endurance training—a [higher] V max and lower HR at rest and during [exerc]ise than the untrained men. Heart volume was [...] larger in T than in UT, a difference similar in [magn]itude to that reported previously between [sedenta]ry controls and highly trained bicyclists [...] 1967) or well trained runners (Ikäheimo [...] 1979). Unfortunately, the X-rays for heart [volu]me determinations for technical reasons had to be done in the standing position. In the lying position, it is not unlikely that the differences between T and UT had been still higher.

A lower maximal HR in endurance athletes than in sedentary controls has been observed in some studies (Saltin & Åstrand 1967) but not others (Pollock 1977). In the present study maximal HR was lower in the very homogenous group of trained men than in the untrained group. However, the cardiac chronotropic reserve of the trained men was not diminished as their resting HR was also correspondingly lower (Fig. 1).

In man, the non-autonomic HR (also referred to as the intrinsic HR) (Sutton et al. 1967) has been studied by pharmacological blockade with competitive parasympathetic and beta-adrenergic antagonists (Jose & Collison 1970, Jose et al. 1970, Nordenfeldt 1971). The question can always be raised whether or not such blockades are complete. In the present study i.v. doses of 0.04 mg/kg atropine and 0.25 mg/kg propranolol or 0.5 mg/kg metoprolol were administered initially. At this dosage level additional doses of atropine or propranolol will not alter submaximal exercise HR (Jose et al. 1970, Lewis et al. 1980). Nevertheless, to ensure complete blockade we added a small additional dose of beta-blocker prior to the second submaximal exercise test. This did not further lower the exercise HR, indicating a complete blockade.

The effects of a cardioselective (metoprolol) and a non-selective (propranolol) beta-blocker (MAB and PAB respectively) included in the autonomic blockade were in most respects not significantly different. HR at rest and at all levels of exercise was the same after PAB and MAB. The reduction of almost 50 beats/min in HR during maximal work after PAB and MAB reflects the equipotency of the two beta-blockers in the doses given (Johansson

[Tab]le 3. *Blood lactate mmol/l*

[...] ± S.D. during exercise [w]ith and without autonomic blockade. Significant ($P<0.05$) difference between non-drug [treat]ment and * Propranolol and Atropine treatment or † Metoprolol and Atropine treatment. ** and †† $P<0.01$

	Group	Non-drug	Propranolol atropine	Metoprolol + atropine
[...]ary	T	0.91±0.69	1.00±0.28	1.15±0.64
	UT	[.]18±1 [.]4	2.13±0.86	2.30±0.95
[...]ary	T	1.84±0.79	[.]95±1.59*	4.04±1.77 ††
	UT	4.90±1.46	5.80±1.89	5.77±1.86
	T	9.23 1.56	5.01±1.55	6.70±[.]43 ††
	UT	11.70±1.65	9.09±2.49*	9.36±1.91 ††

Table 2 Oxygen uptake (V mmol min ¹) pulmonary ventilation (V_E l min) and V_E/V_O2 l and
Mean ± 1 S D during exercise with and without autonomic blockade Significant difference (P<0 05) between non-treatment and Propranolol and Atropine treatment or † Metoprolol and Atropine treatment. and †† P<0.01

	Group	Non-drug	Propranolol + atropine	Metoprolol + atropine
50% V_O max	T	108±12	105±1?	106±1?
	UT	70± 9	65± 9	67±10†
75% V_O max	T	168± 6	160±20*	165± 4
	UT	104±17	100±18	98±13†
V max	T	.26±21	178±?0	189±18†
	UT	139±16	1 1±16	1?6±.3†
V_E 50% max	T	5 .5± 5.8	51.5± 7 6	51 7± 6.9
	UT	47 2± 7 7	41 0± 6.6	43.5± 6.5†
V 75% max	T	84 8±13 6	92 8±17	98 7± 1 4†
	UT	7? 8±1 3	71 0±15..	70.2±11 1
V max	T	151.5±11.5	115 5±10.5	1 7 0±17 6†
	UT	138 6±15 6	108 0±11.8	114.3± 4.3†
V /V_{O_2} 50% max	T	0 49± 0 03	0 48± 0 05	0 49± 0 04
	UT	0 68± 0 11	0 63± 0 09	0 66± 0 11
V /V 75% max	T	0.50± 0 04	0.58± 0 06	0 59± 0 06 ††
	UT	0 70± 0 09	0 71± 0 10	0 7?± 0 08
V /V max	T	0 67± 0 05	0 65± 0 04	0 67± 0 07
	UT	1 00± 0 11	0 90± 0 08*	0 91± 0 09

(Fig 2) During submaximal exercise there was no significant difference in the systolic blood pressure between the trained and untrained men with and without autonomic blockade Systolic blood pressure was significantly lower in each group during exercise after autonomic blockade by the same amount after PAB and MAB (Fig ?)

Pulmonary ventilation

Differences in V_E max between the T and UT groups were not significant under any exercise condition (Table 2) V_E max was reduced under both autonomic blockade conditions in the T and UT groups ($P<0\ 05$) In each group there was no significant difference in V_E max between the different drug combinations (Table ?) After autonomic blockade V_E was slightly lower in the UT group at 50% V_{O_2} max ($P<0\ 05$) and higher in the T group at 75% V max ($P<0\ 05$) compared to the V_E for each group unblocked

The quotient between V and V was unchanged with and without autonomic blockade in T and with and without the metoprolol atropine blockade in UT at maximal work load It was higher in UT at all levels of exercise implying an excess ventilation in this group In both groups the quotient tended to be higher at max work load

Oxygen uptake

V_O max was 3 4 vs ? 0 mmol/kg/min in T and UT groups respectively ($P<0\ 05$) (Table 1). After atropine and propranolol administration a greater reduction of V_O max was observed in the T than the UT (21 vs 13% respectively $P<0\ 05$) (Table 2 and Fig 3) V max was 16 and 10% lower ($P<0$ ... the T and UT groups respectively after atropine and metoprolol administration (Table 2 and Fig 3) During submaximal work V was slightly (1-... mmol/min) but significantly lower after autonomic blockade in both groups (Table ?)

Blood lactate

Blood lactate was lower ($P<0\ 05$) in the T than UT group under each condition after exercise at 50% V_O max (Table 3) Similar results were obtained after exercise at 75% V_O max and ... maximal exercise except that there were no significant differences between T and UT after metoprolol plus atropine administration

After exercise at 75% V max in the T and UT groups blood lactates tended to be higher after autonomic blockade with each beta-blocker than under normal conditions although significantly higher blood lactates were obtained only in the T group (Table 3) After maximal exercise in the T and

… 1977). However, the question of whether or there is a non-autonomic component, i.e. a low-… HR, in the training bradycardia remains … In comparisons of trained and untrained … a lower cardiac rate has been observed in … sinoatrial preparations (Bolter et al. 1973 … et al. 1977) but not isolated whole hearts … & Scheuer 1970, Tipton et al. 1977) of … rats, in trained rats after pithing and dener… (Sigvardsson et al. 1977) and in trained rats … et al. 1976) but not in trained dogs (Tipton … 1977) after combined atropine plus prop… blockade. In man there has been no conclu… evidence that resting HR after full autonomic … is lower after a short period of training. … may be due to the fact that a prolonged and intensive training period is necessary to … the intrinsic HR adaptation that has been … in animals. Training studies are ordinarily … out over a much greater portion of an ani… lifespan than the lifespan of a man. We have … the difficulties of a longitudinal study … by comparing a homogenous group of highly … endurance athletes with a group of un… men.

… data indicate that in humans there is also a … intrinsic HR in trained than in untrained ind… In the present study intrinsic HR at rest … any percentage of V… max was lower in T … UT. This finding was definitely not the … of T being younger than UT, since intrinsic … in man and other mammals decreases with age … et al. 1976, Jose & Collison 1970). Further… HR differences between T and UT were … similar in magnitude—approximately 13 …/min—at rest and during exercise at a given … of V_{O_2} max with and without autonomic …kade (Fig. 1). Thus … non-autonomic compo… in the bradycardia of well-trained men may … to a parallel downward displacement of the … max relationship (Fig. 1).

… significant positive correlations between resting … maximal HR with and without autonomic …ckade (Fig. 4) suggest that a training-induced …ration in the electrophysiology of the sinoatrial … could reset both the resting and maximal HR … lower level. In support of this hypothesis … et al. (1977) found a high positive correla… between the spontaneous frequency and the … frequency response to norepinephrine in … sinoatrial preparations from trained and un-trained rats. Bronson et al. (1976) observed a decreased maximal exercise heart rate and an increased duration of the right atrial monophasic action potential at rest after training in healthy men. They attributed this finding to an altered ionic balance between the inside and outside of the pacemaker cell membrane resulting in an intracellular potassium accumulation.

The non-autonomic component of the bradycardia of well-trained men may also be related to cardiac enlargement. Holmgren & Åstrand (1966) found a significant inverse correlation between maximal heart rate and heart volume in well-trained men and women. Using anthropometric formulas, Ekblom (1969) calculated that the reduction in maximal heart rate after 4 years of training in one subject could be accounted for solely on the basis of a greater heart volume. In the present study heart volume was considerably larger in T than in UT and we found an inverse relationship between heart volume and resting heart rate ($R = -0.64$, $P < 0.01$). Heart volume provides only an overall estimate of cardiac size, giving no dimensional assessment of the various chambers. However, in endurance athletes an increased heart volume determined roentgenographically is associated with ventricular and atrial dilatation measured by echocardiography (Ikaheimo et al. 1979). Although the sinoatrial pacemaker increases its firing rate in response to stretch (Jensen 1971), the tonic stretch which could occur with the atrial dilatation of prolonged endurance training might inhibit the acute stretch response and lower the intrinsic HR (Blomqvist & Lewis 1979).

The mechanisms by which endurance training-induced bradycardia is achieved are thus complex. Furthermore, animal experiments have shown that functioning adrenergic nervous system is necessary for the development of a training-induced intrinsic HR decrease in rats (Sigvardsson et al. 1977).

In conclusion, the present data suggest that after very intense long-term endurance training there is an important non-autonomic component in the bradycardia at rest and at all levels of exercise. This is in contrast to the findings in our earlier study (Lewis et al. 1980) in which we observed no change in the intrinsic HR at rest and during maximal exercise after short-term endurance training, but in accordance with several training studies in animals. In the present study we circumvented the difficulties

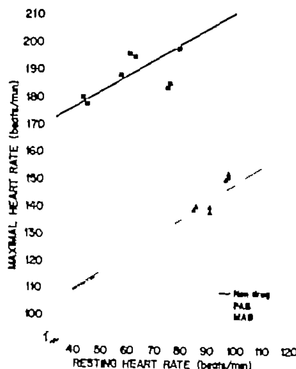


Fig 4 Relation between maximal heart rate during exercise and heart rate at rest in the unblocked state and during autonomic blockade for the trained and the untrained group combined ($n = 16$)

1975) and nearly or totally complete autonomic blockade even at this level of work (Lewis et al 1980) After PAB with smaller doses of propranolol Ekblom et al (1972) found a reduction of 38 beats/min in HR at maximal effort

In the present study resting blood pressure was affected very little by PAB and MAB With submaximal exercise the systolic blood pressure increase was smaller after autonomic blockade probably reflecting a lower cardiac output (Furberg 1968) Systolic blood pressures during exercise were very similar after PAB and MAB which may indicate that peripheral vascular effects which could be expected to be different in a cardio-selective and non selective beta-blocker (Conolly et al 1976) are not the main determinants of blood pressure during exercise after combined vagal and beta adrenergic blockade using the present drug doses At rest without autonomic blockade blood pressure was lower in T than UT in agreement with previous comparisons of athletes and non-athletes (Scheuer & Tipton 1977)

Another difference between a cardioselective and non-selective beta-blocker is the degree of bronchiolar constriction (Conolly et al 1976) However in the present subjects this was not reflected in the pulmonary ventilation which was similar after [illegible] and PAB during submaximal and maximal exercise [illegible] V during submaximal exercise was also [illegible] after MAB and PAB Submaximal V_E and [illegible] however were in most cases slightly but [illegible]cantly lower with than without autonomic blockade [illegible] However at the 75% max load in the trained [illegible] V_E was higher with than without [illegible] blockade Because of the large reduction in [illegible] capacity after blockade in the trained [illegible] was near maximal for several of them

The V_{O_2}/Watt work load tended to be lower after PAB and MAB than in the unblocked condition and the quotient between V_E max and V_{O_2} max [illegible] unchanged with and without autonomic blockade except for a lower quotient after PAB in the UT group (Table 3) which makes it unlikely that the blockades caused an increased work of breathing [illegible] the present subjects Therefore it is also less conceivable that the ventilatory capacity was the limiting factor of the work capacity during PAB and MAB V max was lower after autonomic blockade in both groups In each group the reduction in [illegible] max after PAB tended to be greater than [illegible] after MAB although this was not significant [illegible] PAB and MAB the reduction in V_{O_2} max was greater in the T group than in the UT group (after PAB 21 vs 13% respectively $P<0.05$) In a previous study (Lewis et al 1980) reduction in V [illegible] PAB was similar (18–19% 25–31 ml/min) before and after short term training

The T subjects complained more of extreme [illegible] fatigue in connection with maximal work than [illegible] UT subject This could have been related to [illegible] greater reduction in blood lactate after [illegible] work during autonomic blockade in the T group (Table 3) which could probably explain the [illegible] reduction in V max in T than UT after autonomic blockade (Fig 3) The latter results are in accordance with the findings of Furberg (1968) of a [illegible]creased working capacity in athletes compared [illegible] controls after propranolol blockade He concluded that untrained subjects may compensate for [illegible] cardiac output decrease by an increased peripheral oxygen extraction while athletes already in the [illegible] blocked state have a high a-v [illegible]-difference during heavy work and thus have a smaller reserve [illegible] compensation

Most investigators have attributed the bradycardia of endurance training to an increased vagal [illegible] and a decreased beta [illegible] drive (Scheuer [illegible]

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of a long-term training study by comparing a group of elite athletes with a group of untrained men Possible genetic differences between these two populations (Klissouras 1971) should be recognized and the conclusions therefore viewed with caution The present data demonstrate too that the intrinsic HR at rest and during exercise is very similar when atropine is combined with either the non cardioselective beta-receptor antagonist propranolol or the cardioselective metoprolol

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cal cerebral blood flow in the rat during vere hypoglycemia and in the recovery period lowing glucose injection

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ABDUL RAHMAN A. AGARDH C. D & SIESJÖ B K Local cerebral blood flow in the rat during severe hypoglycemia, and in the recovery period following glucose injection Acta Physiol Scand 1980 109 307–314 Received 13 Nov 1979 ISSN 0001-6772. Laboratory of Experimental Brain Research, E-Blocket the Department of Neurosurgery and the Department of Internal Medicine, University of Lund Sweden.

In order to assess the influence of severe hypoglycemia on local cerebral blood flow (l-CBF) artificially ventilated rats maintained on 70% N_2O were injected with insulin to provide either an EEG pattern of slow-wave polyspikes, or cessation of spontaneous EEG activity for 5 15 or 30 min ("coma) In other animals, glucose was injected at the end of 30 min period of coma and l-CBF was measured after recovery periods of 5 30 90, or 180 min. Local CBF was measured autoradiographically with ^{14}C-iodoantipyrine as the diffusible tracer In the slow-wave polyspike period l-CBF was increased in most of the structures studied, and reached values that were 1 4 to 3.2 times greater than control. In many structures, cessation of EEG activity was accompanied by a further increase in l-CBF with some structures (thalamus, hypothalamus, pontine gray and cerebellar cortex) showing flow rates of 400–500% of control. The increase in l-CBF was unrelated to arterial hypertension hypercapnia, or hypoxia 5 min after glucose injection the hyperemia persisted in only some of the structures studied, in others, the l-CBF were close to or below control values. During the subsequent recovery period l-CBF was markedly reduced with some structures (cerebral cortical areas, hippocampus, and caudate-putamen) showing flow rates of only 20–35% of control In others, notably pontine gray and cerebellar cortex, secondary hypoperfusion was never observed. The hypoperfusion was unrelated to arterial hypotension hypocapnia, or increase in intracranial pressure It is concluded that like hypoxia and ischemia, substrate deficiency due to hypoglycemia is accompanied by vasodilatation in the brain Furthermore, like long-lasting ischemia, severe hypoglycemia is followed by delayed hypoperfusion syndrome that, by restricting oxygen supply may well contribute to the final cell damage incurred

Key words Local cerebral blood flow quantitative autoradiography hypoglycemia

re are reason to believe that the irreversible n damage that may result from prolonged se e hypoglycemia is related to cellular energy fail- Thus, animal experiments have demonstrated when blood glucose concentrations are reduced bout 1 µmol g and spontaneous EEG activi- ceases, the tissue concentrations of phospho- tine (PCr) and ATP are markedly decreased le those of ADP and AMP rise (Hinzen et al b Lewis et al. 1974a Norberg & Siesjö 1976) ociated with this change in cerebral energy state a marked perturbation in concentrations of colytic metabolites citric acid cycle inter- mediates and associated amino acids, probably largely reflecting oxidation of endogenous substrates (Lewis et al 1974b Norberg & Siesjö 1976) It has recently been shown that such a state of pronounced energy failure can be upheld for 30 min and still allow extensive recovery of cerebral metabolic tate (Agardh et al 1978) However in the recovery period there was sustained increase in the lactate concentration and the lactate/

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t 1 Physiological parameters and blood glucose during I ulin-induced hypoglycemia and in the r ry period follow g glucos injection

group consisted of 4 animals, control 10 animals. The values are mean ± S E. MABP= Mean arterial blood pressure

)		MABP (mmHg)	Pa_{CO_2} (mmHg)	Pa_{O_2} (mmHg)	pH	Temp (°C)	Blood glucose (μmol g)
ol		147±3	36 8±1.0	103±1	7.41±0 02	37.0±0 1	6.03±0 40*
ative poly-) EEG		144±8	34 3±1 1	116±5	7.36±0.02	37.3±0.2	1.26 0 11
ctric EEG (min)	5	139±16	34 4±1 1	107_4	7 40+0 03	37.0±0 1	0.73±0.05
	15	151±7	34 1±1.2	103±2	7.34+0 04	37.0±0.3	0.66±0 12
	30	148 8	29 7±3.0*	99±7	7.39±0 03	36.9±0.3	0.42±0 10
ery (min) after m noelectric EEG	5	150±5	38.6±1 9	102±5	7.20±0 05	36 9±0 3	13.30±2 00
	30	145±10	37.0±1 9	114±1	7.25±0 02	37 1_0 1	6 94±0.52
	90	128±1	37.6±4 0	110±7	7.26±0 02*	36.9±0 1	7.26±0 59
	180	111±7*	37.3±_4	101±7	7 19±0 05	37 0±0 2	8.58±0 6_

S E. for the 400 min control animals and the animal injected with insulin and infused with glucose (see text)
$P<0.01$ ** $P<0.001$

riod of ceased EEG activity and in which l-CBF measured either 5 30 90, or 180 min later. Glucose given in an initial dose of 0 5 ml of 50% (w/v) on, followed by constant infusion of the same at a rate of 1 ml h The values obtained were ared to those obtained in control animals maind under otherwise identical conditions (Abdul-Rahet al. 1979) However since the hypoglycemic an were maintained anesthetized for periods of 93–388 two additional (uninjected) control animals were ol hich the period of anesthesia was prolonged min. Furthermore in order to study whether or not rge insulin doses administered had any influence of F tw animals were injected with insulin (40 I U mal, following the maintenance of blood glucose conations above 5 μmol g by slow administration of se solution (1 0 ml of 50% w/v glucose solution in t per hour) l-CBF was measured 400 min later other experiments the intracranial pressure (ICP) was taneously recorded ia cannula inserted in the cister agna during hypoglycemia in animals with an noelec EEG-recording for 30 min and following recovery for (5). The arterial blood pressure was simultane recorded from one femoral artery

Calculations

F as calculated as described in the previous com cation (Abdul-Rahman et al. 1979). Statistical differ between groups were calculated using Student'

RESULTS

le 1 gives the body temperature mean arterial d pressure arterial P_{CO_2}, P_{O_2} and pH as well as d glucose concentrations during and after hypoglycemia. 6 of the control animals were those previously reported (Abdul-Rahman et al 1979) Since physiological parameters between these and the 4 animals studied presently (400 min of anesthesia with or without insulin injection) were not obviously different all 10 animals were pooled The results of Table 1 shows that all hypoglycemic and recovery animal were normothermic and had mean arterial P_{O_2} values of 100 mmHg, or higher Arterial blood pres ure was upheld except in the animals allowed recovery periods of 90 and 180 min in which moderate hypotension developed Arterial P fell moderately after 30 mi of ceased EEG activity but was close to normal in all other groups Arterial pH was upheld during hypoglycemia but was reduced ($P<0.001$) in the recovery groups

Table and Table 3 l st l-CBF values measured in control hypoglycemic, and recovery animals respectively In order to facilitate discussion of results Figs 1 and 2 show values in percent of control during (Fig. 1) and after (Fig. 2) hypoglycemia. As previously stated l-CBF values for 6 of the 10 control animal was reported elsewhere but recalculated according to the autoradiographic standardization and used in the present report. In the two uninjected animal maintained under anesthesia for 400 min, l-CBF values were within the range of these of the 6 original controls. The same applied to one of the insulin-injected animals while the other had some l-CBF values slightly e ceeding this range However since differences were mall

pyruvate ratio and in spite of the fact that the cerebral cortical adenylate energy charge returned to normal values after 3 h the size of the adenine nucleotide pool remained below normal. We speculated that some cell damage must have been incurred, an assumption which has been borne out in a recent histopathologic study (Agardh et al 1980, Kalimo et al 1980).

In order to elucidate the mechanisms by which hypoglycemia leads to cell damage it is important to establish whether energy failure due to substrate depletion is solely responsible or if circulatory factors cause a restriction of oxygen supply as well, adding a hypoxic insult to the primary one. During hypoglycemia this does not seem to be the case. Thus studies in man have shown that cerebral blood flow (CBF) is either normal or increased during hypoglycemic coma (Kety et al 1948, Della Porta et al 1964). In the rat CBF increased both in the slow wave polyspike period and during the first 15 min of hypoglycemic "coma" (Norberg & Siesjö 1976). Furthermore calculations from substrate concentrations reveal that severe hypoglycemia is accompanied by an oxidation of cellular redox systems (Lewis et al 1974b).

Less is known about circulatory events in the recovery period following glucose administration. After shortlasting coma both CBF and cerebral metabolic rate for oxygen (CMR_{O_2}) quickly return to normal values (Della Porta et al 1964). In irreversible coma though both CMR_{O_2} and CBF fall progressively (Fazekas et al 1951). From the results published it is not possible to judge whether the fall in CBF was secondary to a reduced metabolic rate or the cause of it.

As reported in a preliminary communication we have recently observed that sustained hypoglycemia is followed by a progressive reduction in local CBF (l-CBF) during a 90 min recovery period (Siesjö & Abdul Rahman 1979). The present communication provides data for l-CBF in hypoglycemia of sufficient severity to induce slow wave polyspikes in the EEG or cessation of EEG activity for 5, 15 or 30 min, as well as in recovery periods of maximally 3 h following 30 min of hypoglycemic "coma".

METHODS

1. Operative and sampling technique

Male Wistar rats, weighing 275–365 g were starved for 24 h but had free access to tap water. They were then injected with insulin (Novo Actrapid[1], Novo [illegible]) in a dose of 40 I.U. kg i.p. After 60 min the animals were anesthetized with 2.5–3% halothane. When [illegible] spontaneous they were tracheotomized, connected to a [illegible] ing type respirator, paralyzed with tubocurarine [illegible] (0.5 mg kg i.v.) and ventilated on 1% halothane [illegible] 70% N_2O. Respiration was adjusted to give arterial [illegible] values of 35–40 mmHg. Body temperature, as measured in the rectum, was kept close to 37°C by means of a heating bulb.

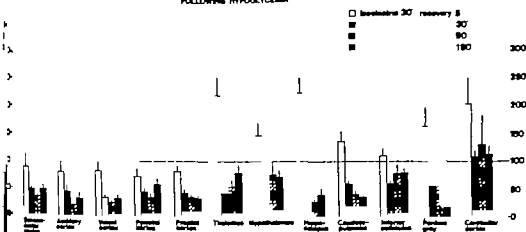
Both femoral arteries were cannulated, one for the pressure recordings and sampling of blood for analysis of pH, blood gases and glucose concentrations, the other for sampling of blood during l-CBF measurements. The latter catheter was cut to a length of 1.5 cm to avoid [illegible] of the arterial ^{14}C-iodoantipyrine curve (see below) [illegible] smearing. One femoral vein was cannulated for infusion of ^{14}C iodoantipyrine and the other one for infusion of glucose during recovery periods. Gold-plated screws were inserted into the skull bone and connected via leads to [illegible] Elema EEG machine. When operative procedures were completed halothane supply was discontinued and the animals were ventilated on 70% N_2O and 30% O_2.

2. Measurement of local CBF

Local CBF was measured with quantitative autoradiography according to Sakurada et al (1978). Details of the procedure have been given in a preceding communication (Abdul Rahman et al 1979). In summary, a solution containing ^{14}C iodoantipyrine (New England Nuclear) [illegible] activity of 40–50 µCi per rat was infused [illegible] ml min. In hypoglycemic animals, arterial blood samples were collected just before and every 3 s following the start of infusion. After 30 s the animal was quickly decapitated and the head was frozen in Freon-1[illegible] at a temperature of minus 50–60°C. In recovery animals blood [illegible] sampled every 5 s and the period of infusion [illegible]. ^{14}C-activity in 50 µl blood samples was measured by [illegible] scintillation counting while the activity in brain was assessed by applying 20 µm sections (at [illegible]°C) to [illegible] film with subsequent microdensitometric measurement. By the use of appropriate standards the tissue activity [illegible] determined allowing calculation of CBF in the structures studied. A careful recalibration of the ^{14}C-standards showed that the values previously assigned to them [illegible] slightly in error. As a result the present l-CBF values [illegible] control rats are about 5–10% higher than those previously published (Abdul-Rahman et al 1979). Details of calibration procedures will be given in another publication (Ingvar, Abdul Rahman & Siesjö 1980).

3. Experimental and [illegible] of groups

Animals were grouped according to the EEG pattern. [illegible] one group l-CBF was measured when the normal [illegible] pattern had been transformed into one with slow [illegible] polyspikes corresponding to a blood glucose concentration of 1.36±0.11 µmol g (mean ± S.E.). Three groups were studied in which spontaneous EEG [illegible] had ceased for either 5, 15 or 30 min (blood glucose concentration of 1 µmol g or lower). The last [illegible] contained animals in which [illegible] se was given after



Local CBF in the recovery period (5, 30, 90 or 180 min) following 30 min period of "isoelectric" hypoglycemia. Values are means ± S.E. in percent of control.

sensorimotor cortex, hypothalamus, pontine and cerebellar cortex. The inhomogenous increase in flow between structures is illustrated by the fact that CBF in hippocampus and cerebellar cortex was 1.4- and 3.2-fold control, respectively.

After 5, 15, and 30 min of severe hypoglycemia (with ceased EEG activity) l-CBF was increased in all structures studied with no obvious relationship to the period of hypoglycemic coma. In most structures the increase in l-CBF was similar to that

Table 3. Local CBF (ml g⁻¹ min⁻¹) during recovery period following glucose injection after a "isoelectric" of 30 min duration

Values are means ± S.E.

Structures	Control 10	Isoelectric EEG 30' and recovery 5' =4	Isoelectric EEG 30' and recovery 30' =4	Isoelectric EEG 30' and recovery 90' =4	Isoelectric EEG 30' recovery 180' =4
Superficial cerebral structures					
Sensorimotor	1.57±0.09	1.–4±0.34	0.77±0.06**	0.55±0.09*	0.77±0.11
Auditory	2.23±0.18	1.79±0.46	0.95±0.24***	0.44±0.02**	0.69±0.20***
Visual	1.35±0.06	1.22±0.29	0.47±0.04**	0.32±0.02*	0.42±0.06 **
Parietal	1.50±0.07	1.10±0.20*	0.69±0.04***	0.53±0.09***	0.88±0.15
Frontal	1.32±0.07	1.08±0.11	0.58±0.05**	0.47±0.07**	0.44±0.06**
Deep cerebral structures					
Thalamus	1.30±0.06	–.90±0.41	1.10±0.11	0.70_0.14 **	1.02±0.16
Hypothalamus	0.98±0.05	1.44±0.21**	0.97±0.14	0.75±0.24	0.71±0.12*
Hippocampus	1.00±0.04	37±0.26**	0.49±0.06 **	0.27±0.03**	0.40±0.10*
Caudate putamen	1.41±0.07	1.86±0.24	0.85±0.06**	0.58±0.07***	0.51±0.06**
Mid brain and pons					
Inferior colliculus	2.28±0.05	2.76±0.32	1.37±0.1	1.78±0.38	1.80±0.15**
Pontine gray	1.10±0.04	1.97±0.37*	1.13±0.08	0.91±0.22	0.85±0.14
Cerebellum					
Cerebellar cortex	1.03±0.05	2.09 0.47*	1.13±0.10	1.35±0.52	1.16±0.14

*$P<0.05$ **$P<0.01$ ***$P<0.001$

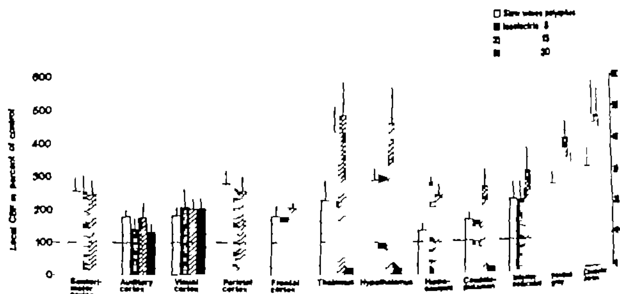


Fig 1 Local CBF during insulin-induced hypoglycemia in animal showing an EEG pattern of slow wave polyspikes, cessation of spontaneous EEG activity ("isoelectric") for 5 15 or 30 min The values are means ± S E in percent of control

all 10 animals were pooled to yield the control group of Table 1

l-CBF during hypoglycemia

Table 2 and Fig 1 demonstrate that during the period of slow wave polyspikes l-CBF was significantly increased ($P<0.01$) in all structures studied except hippocampus ($p<0.05$). Absolute flow rates exceeding 4 ml g⁻¹ min⁻¹ were observed in [illegible] tal sensorimotor and auditory cortex and in [illegible] ferior colliculus In relative terms, flow rates [illegible] ceeding 2 5 times control were observed in [illegible]

Table 2 *Local CBF (ml g⁻¹ min⁻¹) during insulin induced hypoglycemia*
The values are means ± S E

Structures	Control n = 10	Slow wave poly spike EEG n = 4	Isoelectric EEG 5 min n = 4	Isoelectric EEG 15 min n = 4	Isoelectric EEG 30 min n = 4
A *Superficial cerebral structure*					
Cortex					
1 Sensorimotor	1 57±0 09	4 06±0 6[illegible]	4 00±0 78	3 87±0 67*	3 54±0 [illegible]
2 Auditory	[illegible]23±0 18	4 01±0 41	3 17±0 75	4.50±0 54	3 02±0 [illegible]
3 Visual	1 35±0 06	[illegible]50±0 17*	2 8[illegible]±0 70	2.50±0 18*	2.75±0 [illegible]
4 Parietal	1 50±0 07	4 [illegible]±0.58	4 00±0 78*	3 91±0 64	3.79±0 41[illegible]
5 Frontal	1 32±0 07	[illegible]36±0 37*	[illegible]76±0 76	[illegible]75±0 16	3.[illegible]±0 41[illegible]
B *Deep cerebral structure*					
6 Thalamus	1 30±0 06	3 63±0 [illegible]6	5 67±1 01	6 87±1 27*	3 [illegible]
7 Hypothalamus	0 98±0 05	2.87±0 35	3 02±0 63	4 54±1 01	3 36±0.17[illegible]
8 Hippocampus	1 00±0 04	1 38±0 18	[illegible]85±0 49*	3 93±0 98*	[illegible]31±0 2[illegible]
9 Caudate-putamen	1 41±0 07	[illegible]35±0 31	[illegible]90±0 69*	3 76±0 69*	3 11±0 35[illegible]
C *Mid-brain and pons*					
10 Inferior colliculus	[illegible]28±0 05	5 [illegible]1±1.23	5 06±1 [illegible]9*	7 1[illegible] ±1 67	3 70±0 [illegible]
11 Pontine gray	1 10±0 04	[illegible].96±0.38*	4 14±0 66	3 60±0 99*	3 56±0 [illegible]
D *Cerebellum*					
12. Cerebellar cortex	1 03±0 05	3.[illegible]5±0 70	4 63±1	4 85±0.89*	4 [illegible]

$P<0.5$ $P<0.01$ $P<0.001$

...orrelated to hypertension, hypercapnia, and ...us, and previous results have failed to show ...ntra- or extracellular bicarbonate concentra... are reduced (Lewis et al 1974*b* see also ...on et al. 1978, Astrup et al 1978) In view of ...results it appears very unlikely that cellular ...ction of acids could have contributed to the ...mia. Since the increase in CBF was well ...oped already in the period of slow-wave ...ptes in the EEG a period characterized by ...only moderate elevation of extracellular K ...ity (K_e) (Astrup & Norberg 1976) it is also ...ult to ascribe the hyperemia to a rise in K_e. ...ly although it appears probable that adenosine ...l be one of the coupling factors (Berne et al ... the mechanisms of the increase in CBF are at ...nt elusive.

...15 min of ceased EEG activity a few structures ...ed patchy perfusion. Since blood pressure ...normal it seems likely that some localized cell ...a had developed (cf Arieff et al 1973). If so ...edema was transient because in the 30 min ...flow again increased homogenously

...should be emphasized that the secondary ...perfusion during recovery developed at a time ... cerebral energy state is gradually improved ...tissue lactate concentrations are elevated ...rdh et al. 1978). Our results seem to exclude ...ossibility that the hypoperfusion was due to ...dema Thus, although the intracranial pressure ...ased during hypoglycemia, i.e at the time of ...results, this pressure was close to normal in the ...ery period The reduction in l-CBF occurred ...ite of the fact that cerebral perfusion pressure ...maintained at 100 mmHg, or higher and at ...al arterial CO_2 tension These results suggest ...cerebrovascular resistance was increased. ...retically such an increase could be due ...to swelling of endothelial and/or surround... parenchymal cells, or to vasoconstriction ... In view of the fact that circulatory ...tions are similar after prolonged ischemia ...(Siesjö 1977) it would appear important to ...ther explore the mechanisms responsible for ... reductions in flow

The present results demonstrate beyond any ...that at least under the conditions of artificial ...tilation and maintained arterial oxygenation the ...is amply supplied with oxygen even at degrees ...hypoglycemia that extinguish spontaneous EEG ...ity for up to 30 min We conclude from these results that the neuronal lesions that are observed at the end of a 30 min coma period (Agardh et al 1980; Kalimo et al 1980) are the result of substrate depletion alone and should not have been influenced by a concomitant restriction of oxygen supply

The same conclusion obviously cannot be drawn with respect to the final lesions that result from severe hypoglycemia since l-CBF in some structures fell to about 25% of control in the recovery period Our previous results showing a slow but relatively extensive recovery of cerebral energy state after 30 min of hypoglycemic coma (Agardh et al 1978) were obtained on cortical samples from frontoparietal regions. As the present results show CBF in these regions fell to about 30–40% of control after a 90 min recovery period It seems reasonable to assume that this flow reduction retarded metabolic recovery Possibly it could also have contributed to the "final" lesions i.e. to the histopathologic injury that is observed after long recovery periods (Agardh et al 1980 Kalimo et al 1980) However if this is true one must suspect that metabolic recovery is less complete and cell damage more extensive in structures with an even greater reduction in CBF e.g. auditory and visual cortex Clearly it seems highly warranted to further explore the relationship between local blood flow and metabolism as well as the extent of cell damage in various tissue structures

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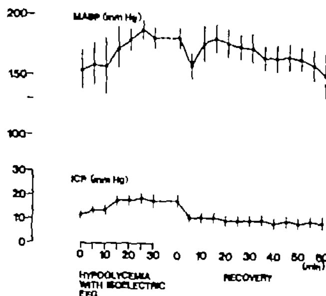


Fig 3 Mean arterial pressure (MABP) and intracranial CSF pressure during insulin induced hypoglycemia (isoelectric EEG for 30 min) and in the recovery following glucose injection The values are means ± S E

observed in the slow wave polyspike period In some of them though l CBF appeared to increase even further following cessation of EEG activity (e g thalamus hippocampus caudate putamen pontine gray and cerebellar cortex) It is note worthy that some structures (e g parietal sensori motor and auditory cortex thalamus inferior colliculus and cerebellar cortex) showed absolute flow rates exceeding 4 ml g min and that some (thalamus hypothalamus pontine gray and cerebellar cortex) had increases in flow to more than 300% of control In a few structures (thalamus hypothalamus and cerebellar cortex) individual animals showed excessively high flow rates with values grossly exceeding 5 mg g min

The data of Table 2 and Fig 1 were obtained by taking repeated densitometry readings from various parts of the same structure in consecutive sections (cf Abdul-Rahman et al 1979) In all groups except that studied after 15 min of ceased EEG activity the flow rates were fairly homogenous within each structure However in the 15 min group l-CBF was inhomogenous in a few structures particularly in parietal cortex In these l CBF could vary from about 5 ml g min down to very low values

Local CBF in the recovery period

The data given in Table 3 and Fig 2 show that 5 min after glucose administration hyperemia persisted in some structures particularly in thalamus hippocampus and cerebellar cortex ($P<0.01$) In structures of the cerebral cortex, though l-CBF values had decreased to or slightly below control value With prolongation of the recovery period l-CBF decreased In most structures, flow rates fell progressively in the period 5–90 min. In some l-CBF was reduced to or below 25% of control (and and visual cortex hippocampus). In others the secondary underperfusion was either small (hypothalamus and colliculus inferior) or absent (pontine gray and cerebellar cortex) Prolongation of the recovery period to 180 min appeared to improve perfusion in some (but not all) structures Thus although the majority of the structures ... flow rates that were grossly subnormal, some had l-CBF values lower than 30–35% of control

Intracranial pressure

The pronounced reduction in local flow rates in the recovery period suggested that capillary circulation could have been impeded by increased interstitial pressure due to tissue edema. However intracranial CSF pressure increased during the period of severe hypoglycemia (i e during the phase of hyperemia) and failed to increase during the recovery period (Fig 3)

DISCUSSION

The present results confirm those obtained with a different CBF technique (Norberg & Siesjö 1976) showing that severe hypoglycemia is accompanied by a marked increase in CBF Our results extend the previous ones to the local level and provide data for coma periods of up to 30 min. In addition they yield information on l-CBF in the recovery period following normalization of blood glucose concentrations at the end of a 30 min period of ceased EEG activity The results demonstrate that like conditions involving a restriction of oxygen supply (hypoxia and ischemia) substrate deficiency leads to vasodilatation in the brain Moreover like ischemia (Hossmann 1977) hypoglycemia is followed by a delayed hypoperfusion syndrome with local flow rates falling to very low values in ... structures studied

The results provide some interesting information on the mechanisms of the hypoglycemic hyperemia and the posthypoglycemic hypoperfusion ... the hyperemia developin during the hypogly...

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Intraglandular transport of [125]I-glandular kallikrein in the rat submandibular salivary gland

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ØRSTAVIK, T. B., GAUTVIK, K. M. & NUSTAD, K.: Intraglandular transport of [125]I-glandular kallikrein in the rat submandibular salivary gland. Acta Physiol Scand 1980, 109: 315–323. Received 15 Nov. 1979. ISSN 0001-6772. Department of Physiology and Biochemistry, Dental Faculty, University of Oslo, the Norwegian Radium Hospital, Oslo, and Department of Physiology, University of Oslo, Norway

The transport of radiolabelled rat submandibular gland kallikrein was studied after local administration to the resting and activated rat submandibular gland. The iodinated kallikrein was electrophoretically, immunologically and biologically indistinguishable from the intact enzyme. After intraductal and intraglandular application the radioactivity in venous effluent was quantitated and characterized. As judged by gel-filtration [125]I-kallikrein in venous effluent eluted at a position similar to that seen when the iodinated enzyme was mixed with plasma, but earlier than the elution of [125]I-kallikrein in buffer. In plasma, therefore, glandular kallikrein is probably bound to macromolecules. The radioactive fractions in venous effluent did not contain free iodine. Maximum concentration of [125]I-kallikrein in venous effluent of resting glands was repeatedly reached about 20 min after intraductal administration. Moreover, the ductal epithelium represented the main permeation barrier since after intraglandular application the maximum venous [125]I-kallikrein concentration was reached almost immediately. In activated gland (parasympathetic and sympathetic nerve stimulation), the venous [125]I-kallikrein concentration was inversely related to glandular blood flow. We conclude that kallikrein present in the duct lumen or in the interstitium is able to reach the circulation, thereby making possible the local generation of plasma-kinins.

Key words: Glandular kallikrein, glandular transport

Glandular kallikreins (E.C.3.4.21.8) are present in several organs including the kidney and in the secretion from these organs (see Nustad et al. 1978b). They are highly specific for their natural substrate kininogen and the biological function(s) of glandular kallikreins can be explained entirely or in part through the formation of lysyl-bradykinin from kininogens. Through the action of lysyl-bradykinin glandular kallikreins probably are important for the regulation of blood flow in exocrine glands, and may also contribute to local permeability changes and the regulation of electrolyte transport (Nustad et al. 1978b).

Kallikreins are localized in the distal tubules of the kidney (Ørstavik et al. 1976) and in the striated ducts of the salivary gland (Ørstavik et al. 1975, Brandtzaeg et al. 1976, Ørstavik 1978, Hojima et al. 1977). The enzyme is predominantly located in the apical part of the cells. This localization indicates a luminal secretory route, which is further substantiated by the presence of kallikreins in saliva and urine. The kallikrein-substrate, kininogen, is present in plasma (Jacobsen 1966*a*) and has also been detected in the lymph (Jacobsen 1966*b*) and urine (Pisano et al. 1978). On the other hand, kallikrein has been detected in kidney perfusates (Roblero et al. 1976) and esterase activity possibly representing kallikreins has been detected in the renal lymph (deBono & Mills 1974). These findings indicate that at least some kallikreins may penetrate the tubule epithelium to interact with kininogen in the interstitial fluid. The subsequent formation of lysyl-bradykinin might cause vasodilatation and increased permeability of the blood vessels found in

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ØRSTAVIK, T B GAUTVIK K M. & NUSTAD K Intraglandular transport of 125I-glandular kallikrein in the rat submandibular salivary gland Acta Physiol Scand 1980 109 315–323 Received 15 Nov 1979 ISSN 0001-6772 Department of Physiology and Biochemistry Dental Faculty University of Oslo the Norwegian Radium Hospital, Oslo and Department of Physiology University of Oslo, Norway

The transport of radiolabelled rat submandibular gland kallikrein was studied after local administration to the resting and activated rat submandibular gland The iodinated kallikrein was electrophoretically immunologically and biologically indistinguishable from the intact enzyme. After intraductal and intraglandular application the radioactivity in venous effluent was quantitated and characterized. As judged by gel-filtration 125I-kallikrein in venous effluent eluted at position similar to that seen when the iodinated enzyme was mixed with plasma, but earlier than the elution of 125I-kallikrein in buffer In plasma, therefore glandular kallikrein is probably bound to macromolecules. The radioactive fractions in venous effluent did not contain free iodine. Maximum concentration of 125I-kallikrein in venous effluent of resting glands was repeatedly reached about 20 min after intraductal administration. Moreover the ductal epithelium represented the main permeation barrier since after intraglandular application the maximum venous 125I-kallikrein concentration was reached almost immediately In activated gland (parasympathetic and sympathetic nerve stimulation), the venous 125I-kallikrein concentration was inversely related to glandular blood flow We conclude that kallikrein present in the duct lumen or in the interstitium is able to reach the circulation, thereby making possible the local generation of plasma-kinins.

Key words. Glandular kallikrein, glandular transport

...dular kallikreins (E.C.3 4.21.8) are present in ...crine organs including the kidney and in the ...tion from these organs (see Nustad et al ...b). They are highly specific for their natural ...strate kininogen, and the biological function(s) ...landular kallikreins can be explained entirely or ...art through the formation of lysyl-bradykinin ...n kininogens. Through the action of lysyl- ...dykinin glandular kallikreins probably are important for the regulation of blood flow in exocrine ...ans, and may also contribute to local permeability changes and the regulation of electrolyte transport (Nustad et al. 1978b)

...llikreins are localized in the distal tubules of ...kidney (Ørstavik et al. 1976) and in the striated ...cts of the salivary gland (Ørstavik et al 1975 ...Rodriguez et al. 1976 Ørstavik 1978 Hojima et al. 1977) The enzyme is predominantly located in the apical part of the cells. This localization indicates a luminal secretory route which is further substantiated by the presence of kallikreins in saliva and urine. The kallikrein-substrate kininogen is present in plasma (Jacobsen 1966*a*) and has also been detected in the lymph (Jacobsen 1966*b*) and urine (Pisano et al 1978). On the other hand, kallikrein has been detected in kidney perfusates (Roblero et al. 1976) and esterase activity possibly representing kallikreins, has been detected in the renal lymph (deBono & Mills 1974). These findings indicate that at least some kallikreins may penetrate the tubule epithelium to interact with kininogen in the interstitial fluid. The subsequent formation of lysyl-bradykinin might cause vasodilatation and increased permeability of the blood vessels found in

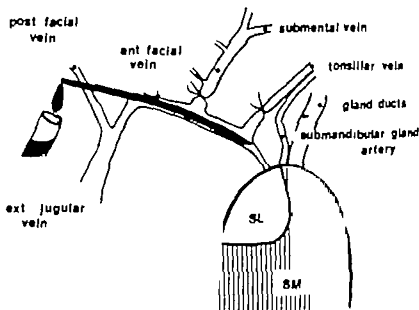


Fig 1 Operating procedure [125]I kallikrein was injected intraductally or intraglandular ly into the submandibular gland The venous effluent was collected by cannulation of the anterior facial vein after occlusion of the submental and tonsillar veins

close approximity to the distal tubules (Beeuwkes & Bonventre 1975) In the salivary glands kallikrein activity has not been demonstrated in venous effluent except when artificial perfusates were used (Hilton 1970 Gautvik et al 1976) However release of kinins into the venous effluent of the dog submandibular gland has been observed following chorda tympani stimulation (Ferreira & Smaje 1976)

In order to examine the intraglandular transport of secreted kallikrein purified rat submandibular kallikrein (Brandtzaeg et al 1976) was labelled with [125]I and injected in microquantities subcapsularly or intraductally into the rat submandibular gland Radioactivity associated with kallikrein was then measured in the venous effluent from the resting and activated gland After application of I kallikrein intraductally or subcapsularly the labelled enzyme was recovered from blood draining the gland

The initial part of this study has been reported elsewhere (Ørstavik et al 1978)

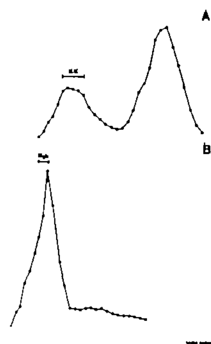
MATERIALS AND METHODS

Animal and operative procedur A t tal of 48 female and male Sprague-Dawley rats aged 3–4 month (250–350 g b wt) were used for the expt The animals were anesthetized with i p injections of pentobarbital (50 mg/kg b wt) They were tracheotomized and had spontan ous breathing during the experimental period All anim received heparin (0 06 ml 5000 I E /ml) injected int left femoral vein before cannulation of the blood vessel but after cannulation of the salivary duct

Cannul tion f the saliv ary duct Cannulation of t ubmand bular gland main excretory duct as perform a described previously (Ørstavik & Gautvik 1977) T was done prior to cannulation of any of the blood vess All glands were cannulated regardless of the applicat route for the iodinated kall krein The ability of the gl to respond to gland activation with secretion of saliva controlled after the completion of the expts Saliva collected in preweighed tubes and the volume was es mated by weight (1 μl = 1 mg)

Procedure f r blood ampling Venous blood from submandibular gland was obtained by cannulation of vein from the ubmandibular and sublingual gland (Fig The cannula (Portex PP50) wa inserted into the ant facial vein prox mal to the submandibular gland bra and pushed close t the branching point of the ton and submandibular gland veins Prior to vein cannul the ton illar vein and the anterior facial vein distal the submandibular gland branch were occluded The ton vein had to be separated from the submandibular gland tery and care wa taken not to provoke bleeding or turb glandular blood supply Occlusion of the latter wa necessary to achieve reproducible results

Blood wa sampled into pre weighed tubes and blood volume w s estimated by weight (1 μl 1 Blood wa collected cont nuously and sampled in p of 1 o mm and blood flow rate thu recorded infusion was given i v thr ugh the right femoral during the experime tal period A Portex pump ulatory peed was used and the infusion rate was



? (A) Gel filtration of Sephadex G75 (20 1 cm) of labelled kallikrein. Free radioactive iodine was rated from the ^{125}I-kallikrein (KK) (B) Gel filtration Sephadex G75 (11 1 cm) of the effluent plasma (50 The radioactivity eluted as one single peak close to moglobin-containing fractions (*HB*). The hemoglobin easily detected by its red color Thus, the radioac measured in the venous effluent did not represent ikrein, but eluted as complex larger than kallikrein.

ding to the effluent blood flow rate from the submandibular gland Donor-blood was obtained by cardiac ture of adult Sprague-Dawley rats, anesthetized and ed with heparin as described above

gland activation was carried out as described previously (Ørstavik & Gautvik 1977). Parasympathetic activation obtained by electrical stimulation of the nerve plexus rounding the main excretory duct of the gland. Sympathetic stimulation was performed by intermittent electrical stimulation of the sympathetic cervical nerve chain.

Iodination labelling of proteins A highly purified rat submandibular gland kallikrein (Brandtzaeg et al 1976) labelled using Na-hypochlorite and ^{125}I (Amersham, land). The reaction mixture contained 0.5 M Na-phosphate buffer pH 7.5 (50 µl), 0 5 mCi ^{125}I (5 µl), 24 µg kallikrein (4 µl), 0 001 N NaClO in 0 05 M Na-phosphate buffer pH 7.5 (10 µl), and $Na_2S_2O_5$ (500 µl of 1 g/l together with 10 g/l KI and 1 g/l bovine serum albumin in 0.01 M Na-phosphate buffer pH 7.5). The free and bound ^{125}I separated on Sephadex G-75 column. The final iodinated enzyme preparation had specific activity of about 4.8 10^{6} cpm/µg kallikrein assuming 75% recovery of the protein. The iodinated kallikrein showed identical behaviour to the original kallikrein on the Sephadex G-75 column in radioimmunoassay (Nustad et al. 1978*a*), and enzymatically

Table 1 *Concentration of ^{125}I-kallikrein i diff rent tiss e and fl ids after intraductal microinjections in one epresentative experiment*

	cpm	% of radioact. applied
Applied ^{125}I-kallikrein (cpm/40 µl)	.3×10^{6}	100
Glandular activity of ^{125}I-KK (cpm/g w w)	2 0 10^{6}	38.9
Maximal concentration in the venous effluent (cpm/ml)	2 9 10^{4}	
Total activity of ^{125}I KK in venous effluent collected during the first 22 min	3.3 10^{4}	14 4
Contralateral control submandibular gland (cpm/g w w)	6.6×10^{4}	2.9
Peripheral blood (cpm/ml)	3 453	
Thyroid glands (cpm/gland)	1 064	<0.1
Saliva (parasymp)		
30 min after intraductal administration (cpm/min/11 µl)	13 000	0 6
33 min after intraductal administration (cpm/min/5 µl)	650	<0 1

Human serum albumin and β-lactoglobulin was labelled with ^{125}I using Iodogen as an oxidating agent (Fraker & Speck 1978) Free and bound ^{125}I was separated on P 100 column. The final iodinated enzyme preparations had specific activity of about 14 10^{6} cpm/µg assuming 75% recovery of the protein.

Measurement of rat submandibular gland kallikrein esterase activity The hydrolysis of α-N-benzoyl-L-arginine ethyl ester (Bz Arg-OEt) was used as measure of kallikrein activity (Nustad et al. 1975).

Administration of iodinated kallikrein Iodinated kallikrein (40 µl 1 3 10^{6} cpm) was applied intraductally or intraglandularly to the submandibular gland. For the intraductal administration (Fig. 1), Hamilton syringe was connected to the polyethylene cannula which was filled to the tip and inserted into the duct. The cannula was fastened with ligature and ^{125}I-kallikrein was infused (1 2 min) into the duct by slight manual pressure. When applied interstitially (intraglandularly), 4 aliquots of 10 µl were injected subcapsularly with Hamilton syringe.

Detection of kallikrein in venous blood and saliva Appearance of ^{125}I-kallikrein in the blood was demonstrated by measurements of radioactivity in venous blood. The radioactive substance in blood was compared to the labelled kallikrein and to labelled kallikrein mixed with plasma in vitro by gel filtration on Sephadex-G 75 column. Total ^{125}I-kallikrein and ^{125}I-kallikrein concentrations in the blood samples were determined Radioactivity in saliva

Table 2 *Effects of intraductal microinjection on epithelial permeability*

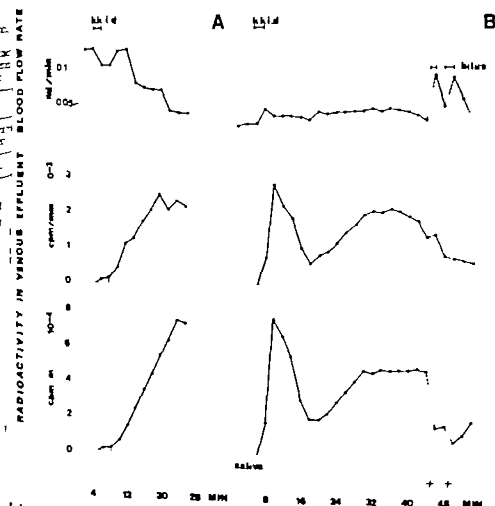
^{125}I-β-lactoglobulin and ^{125}I-albumin were injected intravenously in rats and radioactivity was measured in saliva from both submandibular glands after activations with pilocarpine (4 μg/kg) given i.v. Prior to activation of the glands an intraductal sham injection was performed on one side. Saliva was collected in consecutive periods of 3 min, starting 7 min after the intraductal injection. A total of 27 collection periods from 11 different animals are given. Impairment of the epithelium caused by intraductal application was indicated in only 3 of these rats (+)

Injections			Radioactivity in saliva				Radioactivity in blood
			After intraductal injection		From contralateral control gland		
Proteins	Dose ×10^{6} cpm	Collection periods	cpm/sample	cpm/μl	cpm/sample	cpm/μl	(cpm/μl)
^{125}I-β-lactoglobulin (m w 35 000)	40	I	714	–	723	12	85
		II	428	9	260	12	
	40	I	2 232	13	300	16	75
		II	900	9	859	10	
	40	I	1 574	1	1 575	12	66
		II	579	7	482	8	
	4	I	171	1 6	139	2.2	
		II	71	1.2	73	2.6	
		III	59	1..	48	2.7	9
		IV	51	0 8	56	2.5	
	4	I	49	0 7	57	0.7	25 16
		II	40	–	–		7
		III	35	1.2	38	1.0	
	4	I	165+	1.5+	45	1 1	28 19
		II	77	1 0	72		7
		III	48	1 1	59		1
		IV	51	1 1	–		
^{125}I-albumin (m w 65 000)	40	I	1 18	7	1 220	30	212
		II	747	25	1 021	32	
	40	I	1 083+	26	611	26	212
		II	793	44	708	59	
	40	I	761+	15	510	17	199
		II	500	11	127	9	
	40	I	853	15	1 103	18	185
		II	455	10	574	12	
	40	I	688	10	579	9	
		II	369	10	414	14	

and the gland was counted. For control purposes the radioactivity in the contralateral gland in heart blood and in the thyroid glands were also recorded.

Control of disruption of ductal epithelium after intraductal application. The possibility that the intraductal injections had damaged the ductal epithelium was tested by studying the transport of proteins from blood to saliva. The main duct of both submandibular glands was cannulated. A Hamilton syringe was connected to one of the two glands for intraductal application of buffer as described above. After the intraductal application ^{125}I labelled reference proteins were administered through the femoral vein. 7 min after the intraductal injection pilocarpine (0 4 ml 0 4%) was administered i v and saliva collected from both glands in consecutive periods of 3 min each. Radioactivity was measured in the salivary samples, in blood and in the thyroid and the submandibular glands. Kallikrein apparently combines with components in plasma so that its molecular weight and thus its transport properties may be altered (Nustad et al 1978a). Transport of ^{125}I-kallikrein from blood to saliva may therefore not be comparable to the transport from duct lumen to blood since no binding-substances exist in saliva. Instead β-lactoglobulin (m.w 35 000) and human serum albumin (m.w 65 000) rather than kallikrein were chosen for these control expts. These proteins were labelled with ^{125}I as described above and given in dose equalling 1 1 μg protein (40×40^{6} cpm) and also 0 1 μg protein (4×10^{6} cpm) for β-lactoglobulin.

Histological examinatio ... lands. The effect ...



Intraductal administration of ^{125}I-kallikrein into the resting submandibular gland of two different rats (A and B). Hydrostatic pressure effect of the injection procedure is shown as an initial peak in the concentration of radioactivity in venous effluent (cpm/ml) (B). In spite of this, the gland responded in the regular fashion to parasympathetic stimulation (bilars) at the end of the experimental period with increased blood flow decrease in cpm/ml and rich ...tion. At rest, maximum concentration of ^{125}I-kallikrein in venous blood is repeatedly reached about 20 min after the ...ductal application.

Procedures for ^{125}I-kallikrein application on gland ...phology is controlled after injection of phosphate ...ered saline (PBS, 0.01 M Na-phosphate buffer pH ... 0.15 NaCl) intraductally or subcapsularly. The glands ...re extirpated, fixed in 96% ethanol, brought through ... xylene, and embedded in paraffin. Tissue sections (6 ...) were stained to demonstrate endogenous kallikrein ...the direct immunofluorescence technique as described ...viously (Ørstavik et al. 1975). The tissue sections were ...o especially examined for the presence of endogenous ...likrein interstitially as an indication of disruption of ...tal cells.

Drugs. Pentobarbital (Nembutal sodium® Abbott ...boratories, London), heparin (Heparin Leo 5000 ...ml® Løvens Kemiske Fabrik, Ballerup Denmark), pilocarpine (NAF-Laboratories A/S Oslo Norway) Bovine serum albumin® β-lactoglobulin® and α-N-benzoyl-L-arginine ethyl ester HCL® Sigma, St. Louis, MS USA), Human serum albumin® (ORHA 0405 Behringwerke AG Marburg, W Germany), Sephadex G-75® (Pharmacia Fine Chemicals Uppsala, Sweden), P 100 (BioRad Laboratories, Richmond, California)

RESULTS

Characterization of ^{125}I labelled kallikrein. The iodinated enzyme was homogenous as judged by gel-filtration on Sephadex G-75 (Fig 2 A) The ^{125}I-

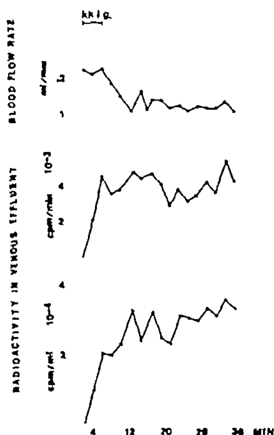


Fig 4 Intraglandular (interstitial) application of ¹²⁵I kallikrein in the resting submandibular gland The concentration of ¹²⁵I kallikrein in venous blood (cpm/ml) increased rapidly reaching a plateau after about ~4 min

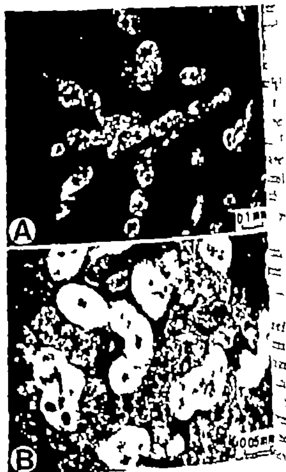


Fig 5 Kallikrein-specific immunofluorescence in the submandibular gland duct cells. When ¹²⁵I-kallikrein administration did not permanently interrupt ductal cell integrity (A), the ¹²⁵I-kallikrein concentration curve resembled that shown in Fig. 3 However an injection that interrupted the ductal cell morphology resulted in a diffusion of endogenous kallikrein (B). In these glands the ¹²⁵I kallikrein concentration curve resembled that seen after an intraglandular administration as shown in Fig 4

kallikrein behaved immunologically (Nustad et al 1978*a*) as the intact enzyme Moreover the iodination procedure did not alter the enzymatic activity of kallikrein since the Bz Arg-OEt-esterase activity of kallikrein (204 ?02 EU/mg protein) was not different from that of kallikrein labelled with ¹²⁵I (208 202 EU/mg protein) The radioactivity in the gland venous effluent after intraductal and subcapsular application was excluded when filtered on Sephadex G-75 (Fig ?B) This is in agreement with the binding of kallikrein to inhibitor-proteins in plasma as seen when ¹²⁵I-kallikrein was mixed with plasma in vitro (Nustad et al 1978*a*) There was only a very small release of free ¹²⁵I from labelled kallikrein since the radioactivity of the thyroid glands was negligible after the experiments (Table 1) and no free iodine was recovered in the venous effluent after gel-filtration (Fig. 2B) Therefore measurements of radioactivity in the venous effluent after injection of ¹²⁵I kallikrein is a useful marker for the intraglandular distribution of kallikrein

Tissue effects of the injection procedure The possibility that the intraductal injections had disrupted the epithelial barrier and thereby given rise to misconceptions was tested in several ways 1 Disruption of the epithelial permeation barrier was recognized by an immediate and high increase in the concentration of venous effluent radioactivity The degree of tissue trauma was indicated by the size of this peak which varied from being absent (Fig 3 A) to a pattern resembling that of an intraglandular administration (Fig 4) Most often a transient initial peak was detected (Fig 3B) Complete absence of this peak ly seen (Fig 3

morphologically recognizable disruption of duc ts and diffusion of endogenous kallikrein were never not observed in tissue sections (Fig. 5 A) ... ments ... th transient initial rise in ^{125}I-kallikrein concentration (Fig. 3B). However when intraductal application of ^{125}I kallikrein produced a ... ntration curve resembling that of an intraglandular administration (Fig. 4) disruption of duc ... l integrity was observed as indicated by gross ... on of endogenous kallikrein (Fig 5B) Also morphologically recognizable changes of the epithelium were present, the glands often re... ded in a normal way to activation (Fig. 3B) ... but the intraductal application did not usually ... ce a permanent damage of the ductal wall ... also demonstrated by studying the transport of ... ted β-lactoglobulin and albumin from blood to ... In eight out of eleven rats the total amount ... the concentration of iodinated protein found in ... as the same in the gland which had re... d an intraductal injection as in the contralateral control gland (Table 2)

These experiments show that the ductal ... elium of these glands was usually not permanently damaged by the intraductal application procedure.

From these experiments it was concluded that the ... file of the ^{125}I-kallikrein concentration curve ... the best indication of the extent of disruption ... the duct epithelial barrier after the intraductal ... administration. Results in electrically stimulated ... ds ... ere disregarded when this concentration ... re indicated a persistent leakage of the ductal ... l.

Transport of ^{125}*I-kallikrein in resting glands* Intraductal microinjection of ^{125}I-kallikrein resulted in ... increase in the specific radioactivity (cpm/ml) in ... enous blood. Maximum concentration was obtained after about 20 min and it remained high for ... the time befor declining (Fig. 3B) In contrast, after intraglandular injections of ^{125}I-kallikrein ... maximum blood concentration was achieved almost immediately (Fig. 4). The results show that ^{125}I-kallikrein is able to penetrate the duct epithelium and enter the blood stream The duct epithelium ... seemed to represent the main permeation barrier since interstitially applied ^{125}I-kallikrein was readily transported into the circulation. The application of ^{125}I kallikrein both intraductally and subcapsularly ... en induced an increase in glandular blood flow (Fig. 3) This increase could be duplicated by saline (data not shown) and was therefore not due to the minute amounts of injected exogenous kallikrein

Diffusion of ^{125}*I-kallikrein i acth ted glands* Parasympathetic nerve stimulation of the gland resulted in saliva formation and increased glandular blood flow (Fig. 3B) Concomitantly there was a parallel increase in total blood radioactivity but a decreased concentration of ^{125}I-kallikrein in venous blood cpm/ml (Fig. 3B) The effect of the parasympathetic stimulation was the same whether the ^{125}I-kallikrein was administered intraglandularly or intraductally Glandular effluent blood flow and the concentration of venous ^{125}I-kallikrein after parasympathetic nerve stimulation were inversely related (correlation factor was −0 70) Stimulation of the sympathetic nerve induced secretion of saliva and a vasoconstriction which was followed by a vasodilatation After both intraductal and intraglandular administration of ^{125}I-kallikrein the stimulation was followed by a parallel increase in blood flow and total venous radioactivity whereas the ^{125}I kallikrein concentration was inversely related to glandular blood flow

DISCUSSION

The purpose of the present study was to examine the intraglandular transport of submandibular gland kallikrein after intraductal and interstitial application. The rat submandibular gland was used since autologous purified glandular kallikrein (Brandtzaeg et al 1976) was available The radioactively labelled kallikrein was immunologically chromatographically and biologically identical to the untreated enzyme When introduced artificially into the duct lumen, the ^{125}I-kallikrein could be used to study the transport of kallikrein within the gland in situ. With the intraglandular application of ^{125}I kallikrein, precaution was taken not to introduce permeability artifacts due to increased intraluminal and interst tial pressure Impairment of the ductal epithelial barrier by intraductal administration of kall krein was best examined by following the appearance of ^{125}I kallikrein in enous blood An immediately occurring peak of varying size in the ^{125}I kallikrein concentration in the venou effluent was believed to represent penetration across the epithelium caused by increased intraductal pressure When this effect was pronounced duct epithelial cells were destroyed and could be detected immunohistochemically by gross diffusion of

endogenous kallikrein from the ductal cells The radioactivity concentration curve then resembled that seen after interstitial application In morphologically unaltered glands the transient increase in ^{125}I kallikrein concentration was followed by a second rise which was comparable in time course and magnitude to that seen when the initial peak was absent This late increase in ^{125}I kallikrein concentration in venous blood which reached its maximum about 20 min after the intraductal injection was believed to reflect the transport of endogenous enzyme across the epithelial barrier

Transport of iodinated proteins from blood to saliva was studied in paired glands 7 min after one of these glands had been subjected to an intraductal sham injection Increased permeation was observed in only 3 out of 11 animals These results further indicate that the ductal epithelium is not usually damaged by an intraductal application In these experiments albumin and β lactoglobulin were used as tracer proteins The much higher ratio between saliva and blood concentration of β-lactoglobulin as compared to albumin probably reflects the difference in molecular weight i e 35 000 and 65 000 respectively since molecules with a molecular weight above 45 000 will be retained also by the basal membrane (Porter & Bonneville 1968) The amount of radioactivity in the submandibular glands did not differ between β lactoglobulin and albumin (data not shown) Thus it does not seem likely that the difference in saliva to blood concentration ratio is due to an accumulation of β lactoglobulin in the submandibular gland

Permeation of radiolabelled kallikrein across an epithelial barrier has previously been shown in the intestine (Moriwaki et al 1973) and salivary kallikrein has been found to penetrate the oral gingival epithelium in an active form causing vasodilatation and periodontal inflammation (Barabash et al 1976) Moreover esterase activity possibly representing kallikrein has been detected in the renal lymph (de Bono & Mills 1974) and kallikrein has been found in kidney perfusate and urine of isolated rat kidneys (Roblero et al 1976) The present study shows that kallikrein may also penetrate the duct epithelium of the rat submandibular gland The ductal epithelium evidently represents the main permeation barrier

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endogenous kallikrein from the ductal cells The radioactivity concentration curve then resembled that seen after interstitial application In morphologically unaltered glands the transient increase in ^{125}I kallikrein concentration was followed by a second rise which was comparable in time course and magnitude to that seen when the initial peak was absent This late increase in ^{125}I kallikrein concentration in venous blood which reached its maximum about 20 min after the intraductal injection was believed to reflect the transport of endogenous enzyme across the epithelial barrier

Transport of iodinated proteins from blood to saliva was studied in paired glands 7 min after one of these glands had been subjected to an intraductal sham injection Increased permeation was observed in only 3 out of 11 animals These results further indicate that the ductal epithelium is not usually damaged by an intraductal application In these experiments albumin and β-lactoglobulin were used as tracer proteins The much higher ratio between saliva and blood concentration of β-lactoglobulin as compared to albumin probably reflects the difference in molecular weight i e 35000 and 65000 respectively since molecules with a molecular weight above 45000 will be retained also by the basal membrane (Porter & Bonneville 1968) The amount of radioactivity in the submandibular glands did not differ between β-lactoglobulin and albumin (data not shown) Thus it does not seem likely that the difference in saliva to blood concentration ratio is due to an accumulation of β-lactoglobulin in the submandibular gland

Permeation of radiolabelled kallikrein across an epithelial barrier has previously been shown in the intestine (Moriwaki et al 1973) and salivary kallikrein has been found to penetrate the oral gingival epithelium in an active form causing vasodilatation and periodontal inflammation (Barabash et al 1976) Moreover esterase activity possibly representing kallikrein has been detected in the renal lymph (de Bono & Mills 1974) and kallikrein has been found in kidney perfusate and urine of isolated rat kidneys (Roblero et al 1976) The present study shows that kallikrein may also penetrate the duct epithelium of the rat submandibular gland The ductal epithelium evidently represents the main permeation barrier

Dr Jørgen Hedemark Poulsen Institute of Physiology University of Copenhagen Denmark gave valuable help in the initial part of this work. We are also grateful to [illegible] Sigurd H From for his valuable advice on the FITC-dextran quantitations and to The Department of Oral Pathology University of Oslo, for making tissue sections for the histological investigations. The study was supported by *The Norwegian Research Council for Science and the Humanities*

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nonary O_2 transfer during pulsatile non-pulsatile perfusion

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HAUGE, A. & NICOLAYSEN G Pulmonary O_2 transfer during pulsatile and non-pulsatile perfusion Acta Physiol Scand 1980 109 325–332. Recei ed 20 Nov 1979 ISSN 0001-6772. Institute of Physiology University of Oslo Norway

The importance of the perfusion pattern for the oxygen transfer has been examined in isolated rabbit lungs perfused with plasma at constant olume inflow The lungs were ventilated with constant tidal olume and constant end-expiratory pressure Following a standardized rise in F_{IO_2} the rate of rise in pulmonary enous P ($\Delta P_{O_2}/\Delta t$) was measured during alternately pulsatile and non-pulsatile perfusion in normal lungs and in lungs made edematous by elevation of left atrial pressure I normal lungs there w no difference in ΔP /Δt when the tw modes of perfusion were compared In edematous lungs ΔP /Δt was statistically higher during pulsatile perfusion, indicating beneficial effect of flow and pressure pulsations, g. a better distribution of V/Q ratios throughout the lungs. I separate series of expts. the advancement of high O_2 front through the airways was measured, and the tw perfusion patterns compared. Since no difference was found, w suggest that the phenomenon of cardiogenic gas mixing in the airw ys is vi is result of direct action of the heart on the lungs rather than arterial pulsations

Key ord Isolated lungs gas mixing, respiration, lung edema, gas xchange diffusion

theoretical effect of pulmonary blood flow ility on gas exchange have been calculated discussed by several workers e g. Crandall & erfelt (1967) Flumerfelt & Crandall (1968) ner et al (1969) Lin & Cumming (1973) and ner & West (1972), all of whom found that flow ility was detrimental to gas xchange Recent owever Bidani et al (1978) pointed out that varying parameters in a physico-chemical sys- in casu pulsatile capillary flow and volume result in improved performance when compared a situation when only one parameter is non- tant ("parametric pumping) They developed mputational model which allowed for such ef- to occur using the pulse contours measured Menkes et al. (1970) Their results suggest that ltaneous pulsatile pulmonary capillary blood and volume can lead to an oxygen exchange is as efficient as in the case of constant flow and me and is more efficient than either pulsatile or pulsatile volume alone

f one turns from the many theoretical treatises to examine the experimental evidence one will find few works which directly compare al eolo-capillary gas e change during pulsatile and non-pulsatile flow conditions. Gillespie et al (1970) perfused one dog lung with pulsatile or non-pulsatile blood flow and measured the rate of uptake of CO and $^{18}O_2$. They found that pulsatile flow increased the uptake of both gases, CO more than $^{18}O_2$. Based on studies of pulsat le vs non-pulsatile dog lung perfusion Clarke et al (1968) on the other hand suggested that O uptak did not change between the two conditions Their data on gas exchange were rather scarce however

In the present work w have monitored venous (effluent) P after standardized rise in F_{IO_2} dur ing alternating pulsatile and non-pulsatile perfusion of rabbit lungs Hemoglobin-induced facilitation of diffusion and other chemical effects due to hemoglobin were excluded from consideration by using plasma as perfusate focusing the attention on pure physical effects f flow pulsatility Our aim was to provide experimental data which could be of help

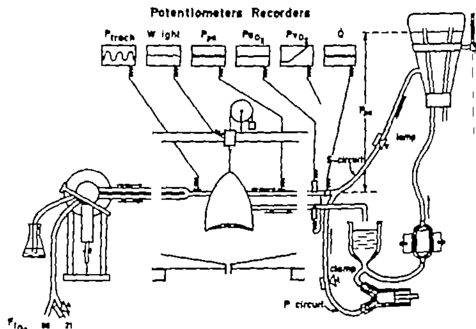


Fig 1 The experimental arrangement for perfusion of isolated rabbit lungs with alternately pulsatile (P-circuit) and steady (S-circuit) flow A detailed description is given in the text P_{trach} = tracheal pressure P_{pa} = pulmonary arterial pressure Pa_{O_2} = pulmonary arterial P P = pulmonary venous PO_2 Q = flow F_{IO_2} = fraction (vol/vol) of oxygen in dry ventilation gas mixture

when the relative merits of the many theoretical studies in this field are assessed

METHODS

Isolated perfused lungs from albino rabbits of either sex weighing 3 0–4 0 kg were used The lung donor animal was anesthetized with pentobarbital (Nembutal Abbott) 30–50 mg/kg i v The procedure for removal of the heart and lungs was as described by Hauge et al (1966) The chest was opened under positive pressure ventilation which was maintained until the circulation was stopped Just prior to the cardiac arrest, heparin (750 IU/kg) was given i v Perspex cannulas were placed in the left auricle and via the right heart ventricle into the pulmonary artery The perfusion and ventilation arrangement is demonstrated in Fig 1

The lungs were suspended underneath a force transducer (Sanborn FTA 100-1) connected to the heart and the cannulas inside a thermostated (38°C) Perspex chamber Lung weight was continuously monitored The lungs were ventilated with constant stroke volume positive pressure and with a stroke frequency from 34 to 38/min The ventilator a Starling "Ideal" ventilation pump I shown schematically to the left in Fig 1 End-expiratory pressure was set with a waterseal as demonstrated and inflation pressure (P_{trach}) was recorded by connecting a pressure transducer (Hewlett Packard Model 70) to a sid branch of the tracheal cannula Equidistant from the inlet of the ventilation pump were positioned two gas-filled balloons one containing air with 4% CO_2 the other 96% O_2 and 4% CO At a standardized point of time in the ventilation cyclus one could by this arrangement, switch from ventila- tion with normal to ventilation with high oxygen frac- (F_{IO_2}) The effect of a change in F_{IO_2} on the partial pressure of oxygen in the effluent plasma (P_{vO_2}) was recorded with a thermostated Radiometer O_2 electrode connected to a Beckman Model 160 oxygen analyzer Membrane thickness was 5 to 6 µm.

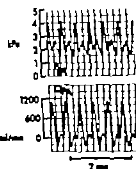
Pulmonary arterial pressure (P_{pa}) was measured via a catheter from a sidearm of the arterial cannula (Statham transducer P23De)

At the start of perfusion left atrial pressure (outflow pressure) was kept at 0–0.1 kPa In the experiments involving hydrostatic pulmonary edema, left atrial pressure elevation was carried out by leading the perfusate coming from the left atrium through the desired position in a vertical ladder of glass and rubber tubing (not included in Fig. 1) the pressure being either 1.3 or [illegible] Zero reference for both in- and outflow pressures was the level of the openings of the perfusion cannulas [illegible] cm below the top of the lungs. The total height of the lungs was about 8 cm

From the thermostated venous reservoir the perfusate could follow one of two alternative routes to the pulmonary artery as shown in Fig 1

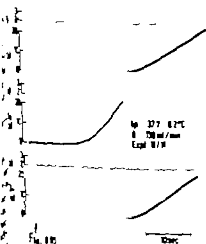
The P-circuit The perfusate could be pumped from the reservoir directly into the pulmonary arterial tubing [illegible] Harvard pulsatile blood pump Model 14?1 The stroke volume and the frequency was adjusted to give a mean [illegible] within the normal range

The S-circuit Alternatively the perfusate could be pumped with a variable speed peristaltic pump (Harvard Model 1210) to a second thermostated arterial reservoir from which it flowed [illegible] ravity into the ar[illegible]



Typical examples of pulmonary arterial pressure ... recordings during pulsatile perfusion.

h. The height of this second reservoir above the ... of the openings of the perfusion cannulas could be ... to give any desired pulmonary arterial pressure ... experiment was always started using pulsatile flow ... gives typical example of the pulmonary arterial ... and flow contours. In order to avoid vasomotion ... uncontrolled variations in vascular resistance 15 mg ... chloride was added to the perfusate. By alter... between the two perfusion circuits, maintaining ... mean flow, the effect of switching from low ... F_{IO_2} could be studied repeatedly during the



3. Three recordings of changes in pulmonary venous (ΔP_{O_2}) following step rise in F_{IO_2} (at arrow) from ... to 0.95. Responses obtained during steady (non-pulsatile) flow are shown in the top and the bottom sections. Response obtained during pulsatile flow is shown in the ... section. Mean flow was kept constant throughout ... expt. The figure demonstrates the differences in the ... of the P_{O_2} curve ($\Delta P_{O_2}/\Delta t$) and the differences in ... time elapsed from the start of high oxygen ventilation ... P_{O_2} begins to rise ("time to take-off"). Also shown is ... concomitant pulmonary arterial pressure (P_{PA}) recordings. During the first part of the pulsatile flow period the ... pressure recording was electrically damped.

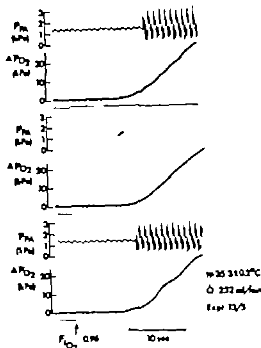


Fig. 4. Three recordings of changes in pulmonary venous P_{O_2} (ΔP_{O_2}) following step rise in F_{IO_2} (at arrow) from 0.1 to 0.96. Responses obtained during pulsatile flow are shown in the top and bottom sections. A response obtained during steady (non-pulsatile) flow is shown in the middle section. Mean flow was kept constant. Also shown are the concomitant pulmonary arterial pressure (P_{PA}) recordings. During the first part of the pulsatile flow periods the pressure recordings were electrically damped.

two modes of perfusion. Flow was measured by flow-through transducer connected to a Nycotron 376 square-wave electromagnetic flowmeter. Pressure, weight, flow- and P_{O_2}-signals were recorded on Hewlett Packard 7402A Oscillograph recorders and on Sanborn Model 320 dual-channel amplifier recorder.

Hauge & Nicolaysen (1971) found that the lung preparation used in the present studies had normal ultrastructure without interstitial edema after several hours of perfusion with pulsatile plasma flow at physiological vascular pressures and normal vascular resistance.

In one expt. the transit of Evans blue through the lungs during pulsatile and non-pulsatile flow was examined. The experimental setup was identical to that described above, except that the total venous outflow from the preparation could be diverted to rapid sampling system (Nicolaysen 1971). By this system the outflow from the lungs could be sampled in split fractions with one sample per 0.67 s. Since the total outflow could be sampled there was no recirculation. Initially pulsatile flow was established. During this perfusion 0.3 ml of solution of Evans blue in horse plasma was rapidly injected into the tubing to the pulmonary artery. The injection was performed in this and the subsequent tests at peak inspiration. Simultaneously

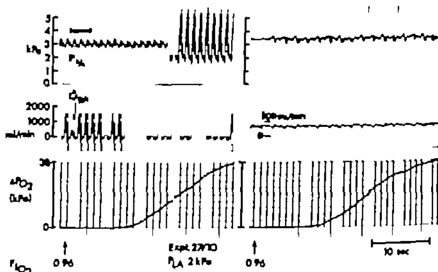


Fig 5 Two recordings of changes in pulmonary venous P (ΔP) following step rises in F (at arrows) from 0 1 to 0 96 in a situation of ongoing transvascular fluid filtration Also shown is the concomitant pulmonary arterial pressure (P_{PA}) and flow recordings (Q̇) Mean flow was kept constant

the sampling of the outflowing pla ma was started The perfusion wa then changed to nonpulsatile and injec tions of Evan blue and sampling of the venous outflow performed Then pulsatile flow wa reestablished and a 4th injection/sampling done The samples of the outflow were weighed and the optical density (O D) at 600 nm determined

In one group of expt designed to tudy airway diffu ion the lungs were not ventilated Transpulmonary pres sure wa maintained constant by connecting the trachea to a pressurized ga tank via a ga flowmeter and an overflow ystem The experiment were done by alternating be tween air (with 4% CO) and oxygen- (with 4% CO_2) containing ga tank The tubings and gas flow c onnec tions were designed in uch a way that ga switching could take place without lung mo ement In this particular series of expt the content of oxygen in the tracheal ga wa measured continuously via a small bore catheter by a Centronic mas spectrometer 200 MGA connected to a Rikadenki recorder

RESULTS

The kinetics of airway to blood oxygen transfer during alternately pulsatile and non pulsatile perfusion was assessed by two variables

(1) $\Delta P_{O_2}/\Delta t$ which is the slope of the middle 1/3 of the recorded P_V range and

( ) Time to take-off which is the time from switching to high oxygen (F_I 0 96) ventilation gas to the point when venous oxygen pressure (P) started to rise

The two variables are shown in Fig 3 which demonstrates a particularly large difference be tween pulsatile and non pulsatile flow both time to "take-off" and $\Delta P_{O_2}/\Delta t$ are considered though the results demonstrated in this record are atypical the figure is included since it with clarity demonstrates what was measured

The P obtained during ventilation with 4% CO_2 was set on the recorder baseline recorded P range above this value was on average 24 kPa (182 mmHg) The shape of the above this range was not considered Fig 4 demonstrate typical results obtained in normal edematous lungs respectively Fig. 5 includes recordings (Q_{PA}) in addition to pulmonary arterial pressure (P) recordings The relatively high pulmonary arterial pressure in the latter example to the elevated left atrial pressure (2 kPa) necessary to induce a hydrostatic type of lung edema

An interesting by-observation was oscillations the venous oxygen pressure The oscillations occur both during pulsatile and nonpulsatile

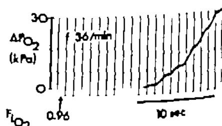
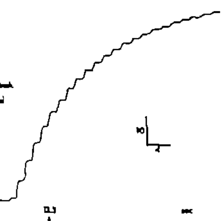


Fig 6 A recording of pulmonary venous PO_2 following a step rise in F from 0 1 to 0.96 during pulsatile perfusion (f=ventilation frequency)



7 The change in tracheal P_{O_2} measured by mass spectrometry following ventilation with gas mixture containing 5% per cent O_2 began at time zero (first arrow), 13.5 ... P_{O_2} as measured with an O_2 electrode ... to rise (second arrow). Tidal volume was 56 ml ... lung capacity 175 ml.

... relatively slow build-up of the tracheal P ... is to ... mainly due to the size of the tidal volume relative to ... internal dead space (up to the gas sampling point).



Fig. 8. Evans blue concentration in pulmonary venous outflow following bolus injection in the pulmonary artery during pulsatile and non-pulsatile perfusion. The bolus injections were performed at zero "accumulated volume". The plasma flow in the four tests was from above: 403, 296, 394 and 331 ml/min.

... in most cases with the same frequency as the ventilation, i.e. from 3 to 36 per min. See Fig. 4 ... tracing and Fig. 6. Such oscillations were dependent on the size of the tidal volume relative to the dead-space of the lungs and the ventilation ..., as well as the dimensions and internal configuration of the connections from the gas balloon to the tracheal cannula. Fig. 7 gives an example of the ... pattern of increase in tracheal P ... following ... rise in F_{IO_2}. Tracheal air was sampled continuously from the level of the bronchial bifurcation and analyzed with mass-spectrometer. The ... volume, the ventilation frequency, the ... space and the gas-connections were similar to ... used during the experiments listed in Tables 1 ...

... Table 1 gives an overview of the results obtained ... non-edematous lungs. Mean flow was kept ... in each perfusion experiment. There was ... systematic effect on pulmonary vascular resistance (PVR) of going from pulsatile to non-pulsatile ... and vice versa. Two pairs of lungs (expt. 1/11 and 17/1...) had relatively high PVR, flow ... 138 and 120 ml/min, respectively. Mean flow ... the other 14 lungs was 236 ml/min, and the average pulse frequency 60 per min. Mean P ... in all the ... listed in Table 1 was 1.46 kPa, average pulse pressure 1.98 kPa, peak inflation pressure 0.48 kPa, and end-expiratory pressure was set at 0.1 kPa.

ΔP /Δt was not significantly different when the two modes of perfusion were compared ($P=0.18$, Sign-test, two-sided). Time to take-off was shorter in lungs perfused with pulsatile flow ($P=0.004$).

Table ... gives the results from lungs with hydrostatic lung edema. Left atrial pressure was elevated to either 1.3 or 2.0 kPa, and extravascular fluid accumulation was monitored gravimetrically. On the average 9.4 g of extra fluid can accumulate in the interstitial space before alveolar flooding occurs (Hauge et al. 1975). The fluid accumulation values listed in the table are those at the end of the expt.

We found without exception that $\Delta P_{O_2}/\Delta t$ was higher during pulsatile than during non-pulsatile perfusion, i.e. the slope of the P ... was steeper. Furthermore we found that in edematous lungs time to "take-off" was always shorter during pulsatile than during non-pulsatile perfusion.

The difference in the time to "take-off" which was observed in both normal and edematous lungs could be due to a shorter blood transit time in the vascular bed, a higher mean linear gas velocity in the airways during pulsatile flow, or both. We de-

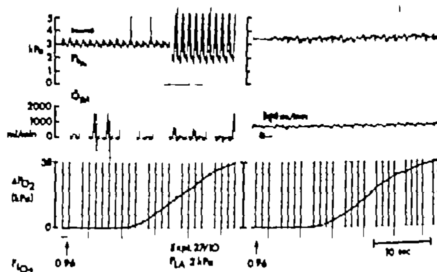


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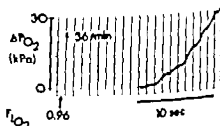


Fig. 6. A recording of pulmonary venous PO2 following a tep rise in F from 0 1 t 0.96 pulsatile perfusion. Fe ventilation frequency

amed the appearance and the concentration ver outflow olume of E ans blue in one perfused preparation (Fig 8) The result indicate that appearance of the tracer and the peak concen are found at the same accumulated outflow me (e enous outflow after the time of injec) hen the two modes of perfusion are com- Since the flow was not exactly the same ng the 4 tests, accumulated volume was used ead of time The range of the olume flow was same during pulsatile and non-pulsatile perfu-

ally we compared the advancement of the oxygen front in inflated but unventilated lungs ng pulsatile and non-pulsatile perfusion At a n point of time the positive pressure inflation source as changed from air (with 4% CO_2) to gen (ith 4% CO_2) and the time which elapsed tracheal P_{a_2} and venous P started to in- se, was recorded The results are listed in Ta- 3. There was no systematic difference when the modes of perfusion were compared under con s of equal olume flow

CUSSION

most important observation made during the first series of experiments was that the gas- sfer in normal (non-edematous) lungs, meas- as $\Delta P_a/\Delta t$ was equally efficient during pul- le and non-pulsatile flow The investigation does lend support to the many theoretical studies ch conclude that flow pulsatility is detrimental as exchange The result is compatible with the introduced by Bidani et al (1978), outlined ve.

edematous lungs pulsatile blood flow was tly more efficient than a non-pulsatile mode of fusion. Several workers have observed that in- titial edema tends to cause flow deviations away n the edematous regions (West et al 1965 hes et al 1968 Iliff et al 1972) The relative e vascular transmural pressure swings during satile flow may well contribute to maintain the ency of the lung vessels in the presence of ede and in this way preserve a more normal allover tilation/perfusion relationship. Iliff et al. (1972) found that interstitial edema tended to cause a in terminal airway resistance and even terminal ay closure The deviations in flow and ventila- were not matched, however If the terminal airway dimensions are better maintained during pulsatile flow the build-up of alveolar P following a rise in $F_{I_{O_2}}$ would be more rapid This would be an alternati e or a complementary explanation of the present findings

In our opinion the measured variable time to take-off" is less informati e and more related to the particulars of the experimental design than $\Delta P/\Delta t$ Clearly the long absolute time which elapsed from the low to the high $F_{I_{O_2}}$ switch until enous P started to rise to a large extent reflected the distances and the dimensions of the external gas connections A main determinant for time to take-off" was therefore the build-up of a higher alveolar P_{a_2}. This does not however explain the statistical difference between pulsatile and non-pulsatile perfusion since the geometry of the experimental set-up was the same Furthermore the difference was present regardless of the occurrence of lung edema

Two sets of experiments were carried out with the purpose of finding an explanation We reasoned that the transit time for plasma from the alveol to the position of the oxygen electrode could be shorter during pulsatile than during non-pulsatile perfusion and/or that the mean linear gas velocity in the airways could be higher due to arteriogenic gas mixing The results from indicator dilution experiments (Fig. 8) made the first of these two hypotheses less probable so we turned our interest to the gas movement in the airways.

During a normal inspiration gas moves through the pulmonary airway by a combination of convection and diffusion. In the terminal airways and the alveolar ducts molecular diffusivity of oxygen is assumed to be sufficiently great in relation to local dimensions to completely mix the inspirate with alveolar gas (Fukuchi et al. 1976) In the larger airways however where linear dimensions are greater molecular diffusion is insufficient, resulting in concentration gradient or front between inspired and alveolar gas. Several studies have demonstrated that the mechanical action of the heart enhances gas mixing in the lung, both during breath holding (Engel et al 1973) and during inspiration (Fukuchi et al 1976) This effect has been called cardiogenic mixing It has been calculated that it increases the effective diffusion coefficient by a factor of five

It occurred to u that we were in the unique position to be able to study the effect of pulmonary

Table 1 *Pulmonary venous P_{O_2} response to a step increase in F_{IO_2} during pulsatile and non-pulsatile perfusion (normal lungs)*

Expt	$\Delta P_{O_2}/\Delta t$ (kPa/s)[a] Pulsatile	n	Non-pulsatile	n	Time to "take-off" (sec)[b] Pulsatile	n	Non-pulsatile	n
5/11	26	8	_5	6	10 4	8	11 6	6
11/11	2.7?	6	1.56	6	10 3	6	?0.5	6
3/1?	3 40	9	90	6	7 0	9	9 8	6
17/12	2.33	8	? 90	6	10 1	8	6 1	6
25/1	3 09	9	3.56	9	8 8	9	10 1	9
27/1	5 68	13	? 68	9	6 0	13	5.8	9
1/3	3 63	8	85	6	6 8	8	1 0	6
3/5	3 67	9	3 83	6	6 1	8	6.2	6
12/5	3 06	9	? 97	9	7 0	9	8 6	9
13/5	_59	9	.59	9	7 8	8	9 8	9
_3/5	? 75	3	46	3	8.3	3	9.5	3
2/6	2 77	6	2 43	6	7.3	6	7 8	6
9/6	1 49		1 88	4	8 9		10 0	4
2_/9	1 01	9	0 80	6	12 6	9	19 0	6
6/10	15	9	1 21	6	1 4	9	13 6	6
20/9	2.31	9	1 73	6	7 0	9	8 4	6

[a] $\Delta P_{O_2}/\Delta t$ is the slope of the middle 1/3 of the recorded pulmonary venous P_{O_2} range Values are the means from [n] observations
[b] The time from switching to a high F_{IO_2} (0 96) to start of the rise in venous P_{O_2}. Values are the mean of [n] observations.

Table 2 *Pulmonary venous P_{O_2} response to a step increase in F_{IO_2} during pulsatile and non-pulsatile perfusion (edematous lungs)*

Expt	$\Delta P_{O_2}/\Delta t$ (kPa/s)[a] Pulsatile	n	Non-pulsatile	n	Time to "take-off" (sec)[b] Pulsatile	n	Non-pulsatile	n	ΔWt (g)[c]
2/6	2 03	4	1 93	4	9 1	4	9 6	4	7
22/9	1 34	3	0 93		10 1	3	18 0	2	3
27/10	62	4	1 86	3	8 3	4	8.4	3	?0
12/11	3 69	4	2 91	4	7 5	4	9.5	4	6
17/11	42	4	2.34	3	8 3	4	8 5	4	10

[a] $\Delta P_{O_2}/\Delta t$ is the slope of the middle 1/3 of the recorded pulmonary venous P_{O_2} range Values are the means from [n] observations
[b] The time from switching to a high F_{IO_2} (0 96) to start of the rise in venous P_{O_2} Values are the mean of [n] observations
[c] ΔWt gives the amount of accumulated extra vascular fluid caused by left atrial pressure elevation

Table 3 *Velocity of O_2 advancement in non-ventilated lungs during pulsatile and non-pulsatile perfusion*

Expt		Time from air/oxygen gas switch till the rise in P_{O_2} trach (s)	n	P_{O_2} (s)	n	Flow (ml/min)
6/9	Pulsatile	4 2±0 45	5	4 3±3 1	6	12
	Non-pulsatile	4.3±0 69	6	2_ 9±3	6	
13/9	Pulsatile	4±0 49	6	19 8±2 1	6	180
	Non-pulsatile	3 0±0 96	6	?0 8±1 7	6	
20/9	Pulsatile	3.8±0 29	4	29 0±2 8	6	300
	Non-pulsatile	4 3±1.33	6	?8 5±1 9	6	
27/9	Pulsatile			23.2±4 3	9	300
	Non-pulsatile			18.3±1 7	6	

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n blood flow in humans as a function environmental temperature measured by ultrasound

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THORESEN M & WALLOE L. Skin blood flow in humans as function of environmental temperature measured by ultrasound Acta Physiol Scand 1980 109 333–341 Received 2 Dec 1979 ISSN 0001-6772 Institute of Physiology University of Oslo Nor ay

W have measured the blood velocities in arteries supplying the skin in humans both in areas with arteriovenous anastomoses (AVA) and in skin areas without AVA in different temperature situations by mean of pulsed bidirectional doppler ultrasound instrument. The instrument measures the time average of the instantaneous mean of the blood velocities present in cross-section of essel So long as the cross-sectional area of the vessel is constant, this average elocity is proportional to olume flow. We observe rapid and large fluctuations in blood elocities in arteries supplying skin areas with AVA in a comfortably warm environment. These fluctuations are substantially larger and more rapid than described by previous authors who have used plethysmograph methods. The blood elocities are more constant both at higher and lower environmental temperatures There are no similar fluctuations in blood velocity in arteries supplying skin areas without AVA.

Key ord Skin blood flow temperature, ultrasound, doppler

l circulation has long been of interest to iologists because of its dominant role in temture regulation (Fredericq 1882) Since each every species has its own unique temperature lation, human skin circulation has to be inigated in humans (Elizondo 1977) This limits possible experimental methods. Temperature surements (Stewart 1911 1912, Lewis 1930) and plethysmograph method (Brodie & Russell 5, Hewlett & Van Zwaluwenburg 1909) have n most widely used.

With venous occlusion plethysmography one can sure blood flow to the extremities or to parts of m, as for instance flow to hands or feet. I the d, the skin circulation accounts for about 60% the total blood flow and even more in warmer ations (Greenfield Shepherd & Whelan 1951) e skin vascular anatomy in the hands and feet is ferent from that in other parts of the body (Gros- 1902) There are great number of shunts be ten small arteries and veins in the dermis, the called arteriovenous anastomoses (AVA) The AVA are nervously regulated and make possible great changes in skin blood flow (Clark 1938 Molyneux 1977)

Earlier authors all agree that the nervous control of AVA plays an important role in temperature regulation (Molyneux 1977 Hellon 1971 Hertzman 1959)

The presence of AVA in different skin areas in humans are as follows, there are AVA in the nailbed of fingers and toes in the fingertip and sole in the palmar aspects of the fingers in the thenar and hypothenar eminences of the hand but not on the dorsum of the hand or fingers (Grant & Bland 1931) I addition AVA have been shown in the external ear (Prichard & Daniel 1956) but not in any other skin area in humans Molyneux (1977) claims that Hoyer (1877) has found AVA on the tip of the nose in humans but such a statement has not been made by Hoyer

The main contribution to the study of skin blood flow in the extremities has been made by Burton & T ylor (Burton 1939 Burton & Taylor 1940) The

vascular pulsations in the absence of the heart Since the branches of the bronchial and the arterial tree run in parallel and in close proximity to each other it was a distinct possibility that the phenomenon of cardiogenic mixing were at least in part due to arterial pulsations In order to keep convective gas movement low the lungs were not ventilated in this series of experiments We found no systematic difference in the velocity of the oxygen front movement from the trachea to the venous blood when the two modes of perfusion were compared Tracheal air was continuously sampled with a flow of 20 ml/min via a capillary cathether From the sampling point to the alveoli however a situation of nearly pure diffusion plus or minus arteriogenic mixing was created If the vascular pulsations enhance the effective diffusion coefficient with a factor of five or even much less a difference should have been detected

Cardiogenic mixing therefore appears to be the result of a direct action of the heart on the lungs rather than an indirect effect via the vascular bed This conclusion is in agreement with Engel et al (1973) whose work indicates "that pulsations of blood vessels next to or beyond the airways did not constitute an important mechanism Although such an accordance in a difficult and controversial field is nice it leaves us without any obvious explanation of the behaviour of the elusive variable time to take-off"

We conclude on the basis of the slope of the pulmonary venous P curve that in normal lungs pulsatile and non-pulsatile perfusion is equally efficient when gas exchange is concerned and that pulsatile flow is of advantage in edematous lungs

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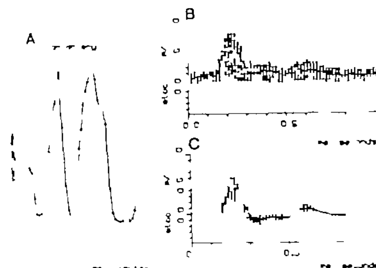


Fig. 2. Fluctuation in blood velocity in the right ulnar artery in 77-year-old male subject in comfortably warm environment (4–28°C). (A) Time average for each cardiac cycle of the cross-sectional mean of the blood velocities in the vessel as function of time. (B) Blood velocities present in the vessel a function of time during one cardiac cycle (marked B in panel A). The instantaneous cross-sectional mean is plotted as continuous line. (C) Blood velocities present in the vessel as a function of time during one cardiac cycle (marked C in panel A).

given at a meeting of The Scandinavian ...iological Society (Thoresen & Walløe 1978).

...THODS

...doppler velocitymeter UNIDOP used in the present ...tigation and the computer systems for data analysis ...been described in detail elsewhere (Hatteland & ...sen 1980, Wille 1977).

...this investigation UNIDOP is used in the pulsed ...le with an operating frequency of 6 MHz. The depth ...ge from 0.2 to 2.5 cm. All the actual arteries are ...ated within this range. The transducer chosen for each ...ry had diameter approximately 1.5 times greater than ...vessel diameter. With this diameter of the transducer ...total cross-section of the vessel is illuminated with ...oximately constant intensity (Gulddvog et al. 1980). ...angle between the direction of the soundbeam and the ...sel was kept at about 45°.

During measurements, the velocity spectra were calculated each 16 ms and displayed continuously in real time ...scope. The instantaneous mean velocity or the time ...rage of this mean velocity over each cardiac cycle ...ld be displayed on storage scope or on an -y plotter ...erfaced to the computer.

The expts. were performed on 6 subjects, 4 males and 2 ...ales (22–40 years) without any known vascular disease. During the expt., the subject was wearing ...imum of underwear and lying in quiet room in one of ...temperature situations, cold, 15–18°C, comfortably ...m, 24–28°C or warm 41–43°C. The air humidity was always low. The subject had been resting in the room for at least 30 min prior to the measurements.

The velocity measurements were performed on arteries supplying different skin areas: the superficial temporal artery supplying the forehead, small artery on the tip of the nose, small artery in the earlobe, the lateral thoracic artery at the height of the nipple, different small arteries supplying the skin of the lower abdomen, the knee and elbow region, the ulnar and the radial artery at the wrist, the radial 3rd finger artery at the basis of the 1st phalanx, the posterior tibial artery behind the medial malleol and the lateral artery of the 1st toe at the basis of the 1st phalanx.

RESULTS

Fig. 2 A shows the average blood velocity for each cardiac cycle in an ulnar artery in a comfortably warm situation. There are three time periods with a rapid fall in average mean blood velocities. We interprete such falls in velocities as corresponding to vasoconstrictions. In two of the periods, the blood velocity decreases rapidly within 3–5 heartbeats to velocity-value only 1/10 of the starting value. The constriction is followed by a somewhat slower dilatation. Panel B in Fig. 2 shows the velocity spectra and the instantaneous mean velocity of one heartbeat on top of the first vasodilatation period,

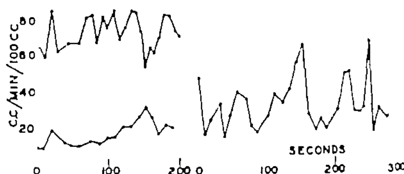


Fig 1 Burton s measurements of fluctuations in the blood flow in a finger in the high and low flow range (plotted to the left) and in the medium flow range (plotted to the right) measured each 10 s by means of a venous occlusion plethysmograph (from Burton (1939) his Fig 7 by kind permission of The American Physiological Society)

skin blood flow in other parts of the human body has not been studied in comparable detail due to the absence of suitable methods

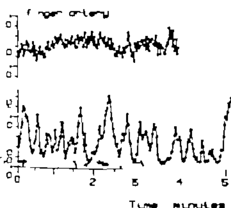
Burton describes fluctuations in blood flow in the finger measured with a finger plethysmograph (Burton 1938) The periodic fluctuations were seen to take place synchronously in all fingers and toes Burton s main findings are displayed in Fig 1 which is reprinted from his paper (1939) In Burton s own words Physical regulation of body temperature is accomplished not by the setting of peripheral blood flow to an appropriate steady level but by the adjustment of the average of a flow which is fluctuating rhythmically between high and low values The rhythm is markedly modified by the conditions of temperature regulation The amplitude of the waves is greatest in the middle range of blood flow and of temperature is less in dilatation and least in constriction The frequency of the rhythm is also modified the faster rhythms being associated with cooler conditions and with lower average values of the flow As shown in Fig 1 10 s elapse between each of Burton s measurepoints By this method it is difficult to increase the time resolution above this value There are also other limitations and difficulties connected with the plethysmograph method Burton (1939) claimed that the minimal and maximal volume flow values in a finger are of the order of 1 and 90 ml/min/100 ml tissue The plethysmograph however cannot measure very high or very low flow values (Abrahamson 1967 Greenfield & Shepherd 1950) Burton s own measurements (Fig 1) indicate a low flow value of 17 ml/min/100 ml tissue compared to a high flow value of 70 ml/min/100 ml tissue

Because of the technical design of the venous occlusion plethysmograph only parts of the extremities have been investigated Consequently there is an almost complete lack of information about the corresponding flow variations in skin areas in other parts of the body

To reinvestigate the skin blood flow pattern in the extremities by a method possessing better time resolution and to extend the skin blood flow measurements onto other parts of the body we have used a bidirectional pulsed doppler ultrasound system developed in our laboratories (Hatteland Eriksen 1980 Wille 1977 Guldvog et al 1980 the ultrasound field emitted from the transducer has a width sufficient to illuminate the total cross section of the vessel with approximately constant intensity the ultrasound equipment measures the instantaneous mean velocity across the lumen of the vessel As long as the cross sectional area of the vessel is constant this mean velocity is proportional to volume flow The last assumption can of course be questioned and we will return to the point in the discussion

With this method we have explored the time course of the fluctuating flow pattern in the skin in greater detail Our findings are mainly in accordance with the results reported by Burton & Taylor (1940) We find however that the fluctuations in skin flow distal in the extremities are substantially greater and more rapid than could possibly have been detected by use of the plethysmograph method In skin areas without AVA on the trunk and head the findings were different. The blood flow to these areas did not show any signs of the flow fluctuations so characteristic of skin areas with AVA

A preliminary report of the results has



5 Blood velocity in an artery to the third finger in 3 ... situations in female subject 26 years old

...plete vasodilation, with high velocity values, only small fluctuations The velocities are 40 ...es greater than in a cold situation.

...ecordings from the posterior tibial the toe and ... finger arteries showed the same increase and the ... pattern of fluctuations with increasing temperature.

...he upper panel in Fig. 4 shows the same three ...ements on the ulnar artery in another subject. ...e subject had a low threshold for sweating, and ...ated profusely during the high temperature ...°C) recording. The fluctuation pattern persisted ... hands and feet throughout the expt and the ...imum velocities in this subject were only 8 ...es greater in a warm situation than in a cold ...ation

The lower panel of Fig. 4 shows tw recordings ... a lateral trunk artery in the same subject. The ... er tracing is from a comfortably warm situation. ...e velocities show no fluctuations The upper ...cing shows recording from a high temperature ...t There are still no fluctuations and the ve...ities are only 3 times greater than in the comfortably warm situation. Velocity values obtained ... the trunk artery in a cold situation, were only ...ghtly lower than in the comfortably warm situation (approximately 0 015 m/)

Other skin areas which do not contain AVA e.g. ...ehead, lower abdomen knee and elbow region, ...played the same nonfluctuating pattern in all ...perature-situations and only a moderate increase in velocities (4–6 times) as the temperature in ... surroundings increased from 18° to 42°C.

Fig. 5 shows recordings from finger artery of third subject in the 3 different temperature situations. This person was sweating only slightly in the warm situation

All our non- weating subjects showed occasional short periods of vasoconstriction in the warm situation Typically there was one vasoconstriction every 3 to 7 min with a 40–50% reduction in a erage mean blood elocity in hand and foot arteries.

Measurement performed on a small artery in the tip of the nose showed the same periodic fluctuations in blood elocities as seen in skin areas with AVA

Fig 6 shows the results from 3 expts. which all illustrate the nervous influence on the vascular bed in skin areas with AVA.

In Fig. 6 A the recording is from a finger artery during a 9 min period The subject came in from the cold with continuous vasoconstriction which ceased as he got warmed and the typical fluctuations appeared

Fig. 6 B shows the result of an expt. on an ulnar artery where the subject puts the opposite hand in cold water for 40 s The a erage mean elocity decreases immediatly to a minimum value and the fluctuating pattern is restored with a delay of 20 s after the hand is taken out of the cold water This rapid response is known to depend upon intact sensory nerves in the immersed hand and on intact efferent sympatic nerves to the other (Lewis 1930)

When similar expts were performed while measurements were being obtained on arteries to the skin of the trunk no change in blood velocities were observed

Several other vasomotor reflexes affect skin blood vessels in the hands and feet (Kunkels Stead & Wers 1939 Abrahamson & Ferris 1940 Burton 1939) The importance of quiet and relaxed environments is shown in Fig. 6C where the subject became aware of a girl watering some plants in the neighbouring hothouse Within a few seconds his ulnar artery showed a rapid vasoconstriction followed by slower vasodilation.

During recordings we often experienced that disturbances such as phone calls or people moving in the room caused vasoconstrictions and stopped the fluctuating pattern in hand and foot arteries Trunk arteries did not show this response

Most of the subjects usually fell asleep during the long experiments in the comfortably warm situation This did not change the cyclic variation in skin blood velocities.

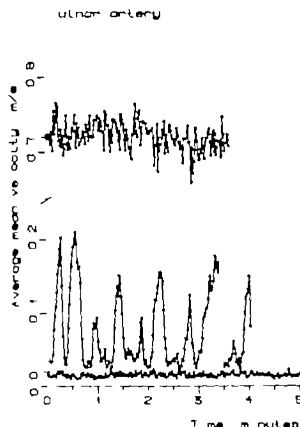


Fig 3 Blood velocity in the right ulnar artery as a function of time in three different temperature situations in the same subject as in Fig 2 Axes and symbols as in Fig 1

marked B in Fig 2A There is a forward flow through the total cardiac cycle and there is a continuous great flow in the diastole Four heartbeats later the average velocity has decreased to 10% and the velocity spectra have changed dramatically as shown in Fig 2C The maximum velocity during systole is only slightly reduced the great change being in the diastole The forward flow in systole is followed by oscillating backward and forward flow during diastole due to the increased peripheral resistance Usually about 80% of the velocity reduction occurred within 3 heart cycles

Fig 3 shows three experiments performed on the right ulnar artery on the same subject in 3 different temperature situations

The lower tracing is from a cold situation the subject lying lightly clothed in 18°C The average mean velocity calculated for each successive cardiac cycle is very low and shows only minor variations. The velocity spectra showed diastolic back flow Such results were always obtained in a cold situation

The middle tracing is typical for medium warm

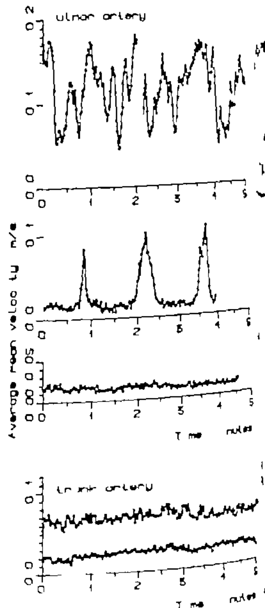
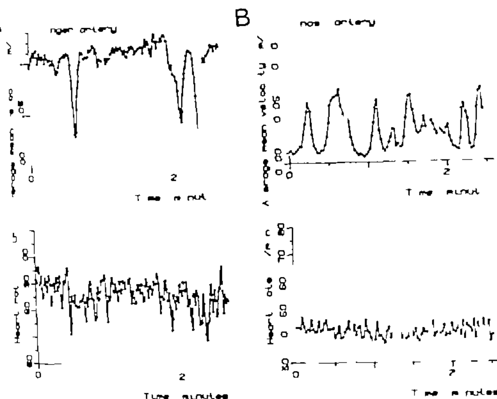


Fig 4 Upper part Blood velocity in the right ulnar artery in three different temperature situations in a male subject 40 years old Lower part Blood velocity in an artery in the skin of the thorax in the same subject in two of the temperature situations shown above (medium and high temperatures)

environments with 2–3 periods of vasodilatation per minute each having a velocity amplitude 5–10 times greater than the minimal blood velocities. As the temperature increases the frequency of vasodilatations usually decreases each period lasting longer. Also the velocity values found during vasoconstrictions increase in value The result is a decrease in the amplitude of the velocity fluctuations

The upper tracing is from an uncomfortably warm situation The subject has rested in a room at 42°C for 2 hours but he is not sweating There



? A, upper panel Blood velocity in finger artery in female subject, 21 years old, in fairly warm environment. ower panel Simultaneous recording of the heartrate calculated by the computer from the ECG recording. Each ac cycle plotted B upper panel Blood velocity in an artery on the tip of the nose in male subject 29 years old comfortably arm environment. B lower panel Simultaneous recording of the heartrate

ss-section of the essel each cardiac cycle. If the ss sectional area of the vessels does not change ring the expt. changes in a erage velocity values proportional t changes in volume flow In pre us work (Guldvog et al 1980 Hange et al 1980) have shown that this condition probably is ful- ed within measurement accuracy in the femoral common carotid artery in the dog and in the ernal carotid and the vertebral artery in man.

What the relationship between the mean velocity d volume flow in the smaller arteries investigated the present study will be is not apparent. There e two possible mechanisms which could change e diameter of the vessels (1) The transmural pres- re of the vessel could alter due to changes in the scular resistance peripherally to the measurement point. (2) The nervous output which affects the ent. (2) The nervous output which affects the sistance vessels could also affect the muscular teries where we are measuring. These two echanisms would probably operate in opposite directions. If the first mechanism is important transmural pressure should drop during periods with vasodilatation The results would be a de crease in vessel diameter and an increase in mean velocities across the vessel lumen greater than cor responding to the increase in flow If the second mechanism is important, the vessel diameter would decrease during periods with vasoconstriction. The result would be fluctuations in verage mean ve locities smaller than the corresponding fluctuations in flow We tend to believe that if either of these mechanisms are of importance it is more likely to be the second one than the first. The reason for this belief is that the vascular resistance between the aorta and some of the vessels where we are measur ing (the ulnar the radial and the posterior tibial arteries) must be small. It is difficult therefore to imagine large changes in transmural pressure in these vessels, when the central blood pressure is constant. Our conclusio is therefore that neither the observed fluctuations in verage mean blood ve-

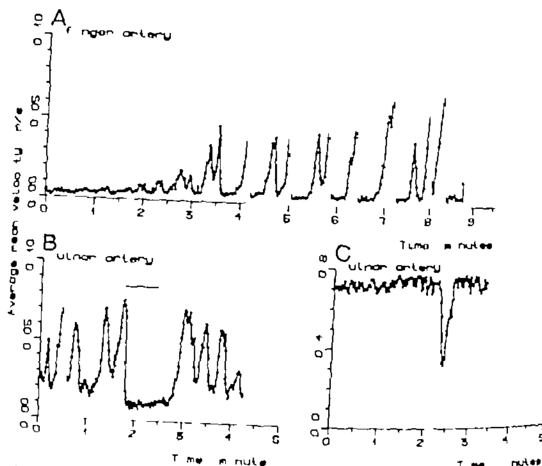


Fig 6 (A) Blood velocity in a finger artery in a period with increasing temperature Male subject 29 years old (B) Blood velocity in the right ulnar artery in a male subject 26 years old in a medium temperature situation The subject put the opposite hand in cold water for 40 s (C) Blood velocity in the right ulnar artery in a male subject 27 years old in a warm environment The vasoconstriction is caused by the sight of a girl in the neighbouring hothouse

Measurements performed simultaneously on the two radial arteries of one subject showed syncronous fluctuations as described by Burton (1939)

In one experiment on one of us (L W) we measured the radial intraarterial blood pressure simultaneously with measuring blood velocity on the opposite 3rd finger in a comfortably warm situation In contrast to Burton we did not find any systematic changes in the blood pressure corresponding to changes in velocities

DISCUSSION

The quantitative knowledge of skin blood flow is mainly based on plethysmograph measurements of limbs and appendages which can be enclosed in a plethysmograph In the digits the flow goes primarily to the skin and Burton & Taylor s work (Burton 1939 Burton & Taylor 1940) on finger blood flow has supplied most of the information about the amount and variability of skin circulation

In the main our findings confirm Burton s work However we find more rapid and greater variation in the blood flow to the hands and feet probably due to the higher time resolution and flow range resolution obtained by our method

In certain skin areas mentioned in the introduction there are arteriovenous anastomoses which permit a great blood flow to pass directly from arteries to veins if they are open We find that the skin areas displaying a fluctuating pattern in blood velocities coincide with the anatomical distribution of AVA The correlation is better than that presented in the figures for example there are no fluctuations in small arteries on the dorsum of the hand

As far as we know no anatomical investigation have documented AVA in the tip of the nose (Clara 1938) However our results suggest that there must also be AVA in this skin area since we observed fluctuating velocity pattern which changes with environmental temperature (Fig 7B)

We measure the average velocity through

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locities nor the changes seen in velocities from one temperature situation to another overestimate the corresponding fluctuations and changes in flow

Measurements of the ulnar finger posterior tibial toe and nose arteries show rapid fluctuations in skin blood flow in a comfortably warm situation The maximal frequency of fluctuations which we have observed is 6 per minute The frequency decreases as the temperature increases With increasing temperature the pattern shifts from that of periodic vasodilations on a steady vasoconstriction to periods of vasoconstrictions on a high flow vasodilator situation

The fluctuations are rapid The average mean velocity may decrease to 10% within 3–4 heart cycles (Fig ?) and the amplitudes are 3 times greater than Burton (1939) describes them

The amplitudes get smaller as the temperature increases This phenomenon was also described by Burton (1939)

The 6 subjects we have investigated showed two different patterns of responses to high temperatures The non sweating or slightly sweating persons increased their average mean velocity in hand and foot arteries to ?0–40 times the values obtained in a cold situation They also showed an almost complete cessation of the fluctuating pattern

On the other hand in persons who sweated profusely the fluctuating pattern sustained and there was only an 6–8 times increase in the average velocity values Thus the pattern of velocities found in the warm situations (42°C) showed considerable differences between persons Each person was however consistent in his or her reaction to different temperatures This interindividual variation probably corresponds to the substantial individual variation in sweating patterns described by Heitzman (1957)

Anatomical investigations show AVA to be present in the human ears (Prichard & Daniel 1956) Because of practical difficulties in obtaining measurements on the small arteries in the ear lobe it has not been possible to record for sufficient periods of time in all temperature situations On the basis of a few recordings our impression is that the ear is the first part of the skin surface to respond with vasodilatation when the person is in the need of heat loss. Thus there seems to be only a small temperature zone where there is a fluctuating flowpattern in the ear

The skin of the head trunk and proximal parts of the limbs do not contain AVA The flow response in these skin areas to different environmental temperatures is quite different from the responses in the hands and feet The pattern is non-fluctuating in all temperature situations In a comfortably warm situation the velocity values measured on a trunk artery are usually not more than twice the values obtained in a cold situation In the heat stress situation the velocity values increased not more than 3–5 times as compared to the comfortably warm situation The non-sweating persons again show the greatest increase This moderate increase is in contrast to the great blood flow values during sweating given by others (Roddie Shepherd & Whelan 195 Greenfield 1963)

The plethysmograph has great limitations in low flow situations (Greenfield & Shepherd 1950). Burton claims that vasoconstriction is accompanied by a simultaneous increase in the cardiac rate (Burton 1939 his Fig 8 C and 1?) When the plethysmograph fails he then uses the periods of cardiac acceleration as a measure of the number of vasoconstrictions We have not in any of our experiments been able to confirm Burton s claim Indeed we have not found any systematic relationship between vasoconstriction and heart rate at all Two examples are shown in Fig 7 Fig 7A displays the velocities in a finger artery in a fairly warm environment and the corresponding heart rate There are fluctuations in the heart rate but they are not correlated to the three vasoconstrictions in the finger artery

Fig 7B shows the velocities in an artery on the tip of the nose in a comfortably warm environment and the corresponding heartrate In this person the heart rate is much less variable There are no correlation between changes in velocities and heartrate

Neither have we found systematic changes in skin flow connected with respiration as claimed by Burton and others (Burton 1939 Burch Cohn Neumann 194) Finally we found no such systematic changes in blood pressure corresponding to vasodilation and vasoconstriction as claimed by Burton (1939)

It appears from our findings that the fluctuations in blood flow to skin areas with AVA are not correlated to any other cardiovascular parameters or events observed It appears probable that the marked fluctuations are related to synchronous openings and closures of AVA

a Physiol Scand 1980, 109: 343–344

w threshold facilitation of inspiration / lung volume increments

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pify mediated, olume dependent excitatory ef n on phrenic nerve activity ha e been recently cribed in anesthetized dogs and pigs but could be ascertained in rabbits and baboons (Bartoli et 1975 Hutczak et al 1977 Cross et al 1980 zarewski et al. 1980). Results presented here on t confirm and further characterize a olume de- dent facilitatory effect on inspiratory intercostal l phrenic activity in this species

a ll pentobarbitone anesthetized cat the mov verage of mass activity of phrenic (Phr) and ernal intercostal (EI) nerves was recorded simul- eously with tidal olume (V) tracheal pressure airflow (V) and end-tidal P_{CO_2}. The effects of nges in lung olume P and V on the Phr and EI jectories were studied both during spontaneous athing and after paralysis w th gallamine while ificially entilated. Lung movement were varied elucidated for a single breath by means of a ume dri en servo-respirator" operated at dif ent gains (volume/phrenic ratio) or by phrenic gered inflations of different time courses and plitudes and, during spontaneous breathing by cheal occlusion at end-expiration. Control and t trajectories were compared at the same level of piratory drive

In lightly anesthetized cats increments in lung ume produced a facilitatory effect on both Phr d EI. Since this effect was completely abolished bilateral cervical vagotomy it apparently did not volve chest wall receptors. The facilitatory re- onse, expressed in terms of the arithmetic differ ce in amplitude of test and control trajectories Phr and ΔEI respectively) was linearly related

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to the corresponding difference in lung olume (ΔV) throughout the in piratory phase but was not simi- larly related to changes in flow (ΔV̇). This is evident from Fig. 1 where ΔPhr and ΔEI are seen to in- crease progressively w th increasing ΔV during in- spiration while the difference in flow remained con- stant. Fig. 1 further illustrates that the responses appear at volume and pressure changes well below eupnoeic V and P

Fig. show that this volume dependent facilita- tory effect could also be demonstrated at zero values of V occurring in the inspiratory phase Two

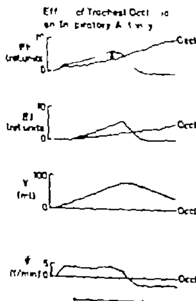


Fig. 1 Inspiratory facilitation during spontaneous breath- ing. From above "Integrated" phrenic (Phr), external intercostal (EI) activity volume (V) and flow (V̇). The computer average of 5 test trajectories (tracheal occlusion at end-expiration) is superimposed on the preceding con- trol trajectories. Five breaths elapsed between each con- trol/test pair. 2 represent ΔPhr (see text for further ex- planation).

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In 11 pentobarbitone anesthetized cats, the mov g average of mass activity of phrenic (Phr) and ternal intercostal (EI) nerves was recorded simul neously with tidal volume (V) tracheal pressure), airflow (V), and end tidal PCO_2. The effects of anges in lung volume P and V on the Phr and EI jectories were studied both during spontaneous eathing and, after paralysis with gallamine while ificially ventilated. Lung movements were varied eliminated for single breath by means of a renic driven servo-respirator operated at dif rent gains (volume/phrenic ratio) or by phrenic iggered inflations of different time courses and nplitudes and, during spontaneous breathing, by acheal occlusion at end-expiration Control and st trajectories were compared at the same level of emical drive.

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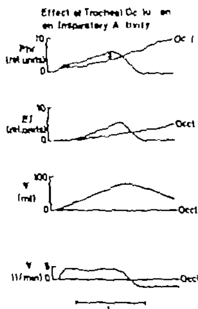


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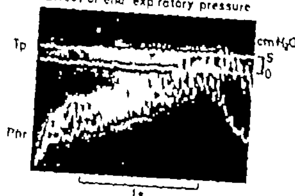


Fig 2 Effect of positive shift of tracheal pres ure Tw superimposed phrenic trajectorie each while the respirator wa switched off Lower trajectory at zero tracheal pressure and upper at +5 cm H_2O Several normal breaths elapsed between traject ries

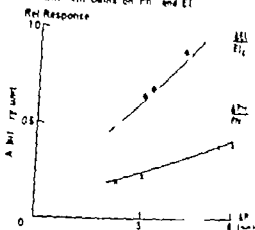


Fig 3 Maximal response values relat e to phrenic and external intercostal control activity (Phr EI) plotted again t changes in tracheal pressure

phrenic trajectories during breath in which the servo-respirator" is turned off (gain 0) are compared at zero pressure (lower curve) and with constant positive pressure applied in the preceding expiratory phase and maintained throughout the inspiratory phase (upper curve) Pres ure increments during the expiratory phase had no excitatory effects ΔPhr remained almost constant independent of inspiratory time and increasing Phr

A linear stimulus/response relationship wa also obtained by changing the gain of the servo-respirator to produce different volumes and relating the maximal respon e value of ΔPhr and ΔEI between breaths to the simultaneous ΔT For purposes of comparison between the phrenic and intercostal responses ΔPhr and ΔEI were normalized to Phr and EI trajectories at a gain setting which produced a V_T similar to eupnoeic breathing As seen in Fig 3 any given ΔP produced a larger perc ntage change in ΔEI/EI control compared t ΔPhr/Phr control Similar results were obtained using phrenic triggered inflation of different shapes and d lays in onset With this procedure it was further demonstrated that V per e did not act as an excitatory stimulu for thi response

Thi volume dependent inspiratory facilitation was consistently decreased or abolished by mall additional doses of pentobarbitone (2–4 mg/kg) After vagotomy the effect could be reproduced in Phr and EI by electrical vagal stimul tion at similar threshold strengths required to produce shortening of inspiratory duration (i e the classical Hering Breuer reflex)

From these result we conclude that low threshold lung mechano-re eptors which mediate volume related information can facilitate inspiratory activity during eupnoeic breathing The stronger facilitatory effect exerted on intercostal motoneurons compared to phrenic may function to progressively increase the relative contribution of the intercostal muscles to the production of tidal volume an effect which would increase in importance as higher tidal volumes are demanded

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Acta Physiol Scand 1980 109 345–354

In vitro studies on a two-pool storage of adrenaline and noradrenaline in granule material from bovine adrenal medulla

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UVNÄS B & ÅBORG C H In vitro studies on a two-pool storage of adrenaline and noradrenaline in granule material from bovine adrenal medulla. Acta Physiol Scand 1980 109 345–354 Received 1 Nov 1979 ISSN 0001-6772 Department of Pharmacology Karolinska Institutet, Stockholm, Sweden.

Adrenal medullary granules were isolated by millipore filtration according to Poisner & Trifaro (1967) and then lysed in deionized water In a perfusion system the influence of concentration and pH was studied on the uptake of biogenic amines (PhEA, TA DA, NA A, Ach and H) and sodium ions by lysed and by dialyzed material The results suggest a two-pool storage of A and NA in the granules. A minor pool with cation exchanger properties binds unselectively organic (biogenic amines) and inorganic cation with U_{max} of 400–500 nmol/mg granules dry-weight This minor pool (pool 1) was fully charged at amine concentrations >10 mM. A larger pool binds selectively A and NA—possibly stereospecifically L-NA and L A—with U_{max} ~1 500 nmol/mg granules. This larger pool (pool 2) required A and NA concentrations ~200–300 mM for maximal filling. In pool 2 CA ions are assumed to be electrostatically linked to carboxyl groups, which become available as hypothetical COO^----- H_3N salt bridges are successively forced open by increasing CA concentrations (>10–30 mM). ATP^- ions become attached to the concomitantly unmasked NH groups.

Key words Adrenal medullary granules, storage of catecholamines, noradrenaline adrenaline adrenal medulla

Bovine adrenal medullary granules have a high content of adenosine 5-triphosphate (ATP) and it was early suggested that the positively charged adrenal catecholamines (CA) were stored ionically bound to the negatively charged polyvalent nucleotide (Blaschko et al 1956, Falck et al. 1956) In vitro CA and ATP have a tendency to aggregate into high molecular complexes—especially in the presence of alkaline earth metal ions (Ca^{2+} and Mg^{2+}) Berneis et al (1971) proposed that the formation of mixed high molecular weight aggregates with ATP plays an essential role in the storage of CA in adrenal medullary granules Various other more or less defined nondiffusible storage complexes containing ATP Ca^{2+} and Mg^{2+} proteins and other macromolecules have been suggested (for ref see Green 1976, Smith & Winkler 1972 Stjärne 1970 and others).

The histamine storing mast cell granules contain no appreciable amounts of ATP but have a high content of heparin Since histamine and heparin form a rather stable salt in vitro histamine was assumed to be stored in the mast cell granules as a histamine–heparin salt However in a series of papers (for ref see Uvnäs 1977) we have presented experimental evidence to show that the mast cell histamine is stored not as a heparin–histamine salt but in ionic linkage to protein carboxyl groups in a heparin–protein complex that forms the matrix of the granules. This granule matrix has the properties of a weak cation exchanger and the release of histamine during the exocytotic process in the mast cell is the result of a cation exchange between the histamine linked to the granule matrix, and extracellular cations, especially sodium ions

A storage and release process due to cation exchange should be a simple and thereby attractive way of explaining the storage and release also of other electrically charged granule stored components In a previous paper (Uvnäs & Åborg 1977)

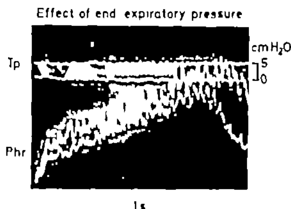


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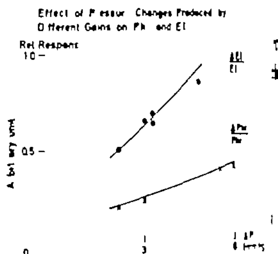


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This volume dependent inspiratory facilitation was consistently decreased or abolished by small additional doses of pentobarbitone (2–4 mg/kg) After vagotomy the effect could be reproduced in Phr and EI by electrical vagal stimulation at similar threshold strengths required to produce shortening of inspiratory duration (i e the classical Hering Breuer reflex)

From these results we conclude that low threshold lung mechano-receptors, which mediate volume related information can facilitate inspiratory activity during eupnoeic breathing The stronger facilitatory effect exerted on intercostal motoneurons compared to phrenic may function to progressively increase the relative contribution of the intercostal muscles to the production of tidal volume an effect which would increase in importance as higher tidal volumes are demanded.

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Fig 1 Electronmicroscopic pictur of granule sediment 9000 g for 10 min. Note the rather homogenous collec- ion of electron dense membrane-surrounded granules. 1365

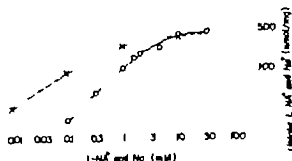


Fig Concentration dependent uptak of L NA and N by dialyzed granule material with concentrations <30 mM in perfusion fluid, pH 7.4 4°C Values taken from Uvnä & Åborg (1977) to illustrate uptake by cation changer pool (pool 1 in the present paper). — L-NA uptake O——O N uptak

the solution The samples er then dried (vacuum, KOH P_2O_5) The rest of the L-NA–ATP salt solution staining granule material as put on membrane filter (0.25 mm Diaflo

Initially 35 and then 900 KPa psi N_2 gas was applied on a granule suspension Each now contained 200 μmol of NA and 90 μmol of ATP per ml.

When the solution had passed the filter and only moist ter remained, this was scraped off (stainless steel spatu- l) into prewighed tube with stopper The wet weight of he material was determined and then the dry weight (after vacuum, NaOH, P_2O_5, 48 h).

To the dried samples and their dried references ere added 1 ml of 1 M HCl (and a few glass beads) After homogenization by shaking and precipitation of undis- solved material at 3000 × g for 30 min, 0.5 ml samples of he supernatant ere taken for scintillation counting Packard, Tri-carb model 2002)

The eighing procedures described above allowed cal- culations of the net uptake of L-NA and of ATP per mg of the dried granule material with corrections for the amounts in the moisture of the granule mass

Influence of pH on he uptake of sodium ... ATP as cation

... μmol of NaOH ere titrated with ATP (acid) 0.2 ... to pH 7.26 as described above 0.25 ml of ^{14}C-ATP (50 μCi/ml) and 0.25 ml of ^{22}Na (200 μCi/ml) were added and ... deionized H_2O added to .5 ml 1 ml of sodium–ATP salt solution and 1 ml granule suspension ere mixed and the procedure continued as described above for L-NA–ATP salt. Final concentrations Na 200 mM ATP 90 mM

... concentration dependent uptake of NA

(a) A *hydrochloride* L-NA hydrochloride was freshly prepared by neutralising L NA base (2.538 g)—with an admixture of 0.1 ml of 5-6% H_2SO_3 and 15 ml of H_2O— with concentrated (12 N) HCl The volume was adjusted to give 600 mM L-NA hydrochloride Final pH was 6.18. The L-NA hydrochloride solution was diluted with H_2O to give 9 samples of 600 400, 300, 200 100, 60 20, 6 and 2 mM L-NA hydrochloride solution. T 5 ml samples of these dilutions were added 25 μl of ^{3}H-L-NA (50 μCi/ml) and 25 μl of ^{36}Cl (NaCl) (90 μCi/ml) Out of each sample 1 ml was taken and mixed with 1 ml of dialysed granule material The final concentrations of L-NA in the samples then were 300, 200 150, 100 50 30, 10, 3 and 1 mM The further procedures ere as described abov

(b) *As phosphate* L NA phosphate was freshly pre- pared by neutralising L NA base (2.538 g)—with an ad- mixture of 0.1 ml of 5-6% H_2SO_3 and 15 ml of H_2O—with concentrated (15 M) H_3PO_4 The olume was adjusted to give 600 mM L-NA phosphate Final pH 6.14. Dilution with H_2O sampling and admixture of H-L NA and $^{32}PO_4^{3-}$ as above with L NA hydrochloride

(c) A *ATP salt* ATP (acid) was freshly prepared as described above and titrated to pH ~6 with L NA base The L NA–ATP salt solution was diluted with deionized H_2O to give 600 mM solution The dilutions and the fur ther procedures were as described above for L-NA hy drochloride

Uptake of PhEA, TA DA, L NA and DL A, Ach H and Na as determined as described for the uptake of L NA above If not stated otherwise all uptak values are given as nmol per mg dried material with deduction of the CA and ATP content

T minimize possible disturbing effects of arious enzymes, of bacterial growth of spontaneous decomposi- tions etc. all procedures described above except titra- tions were performed in the cold room (4°C) An Agla micrometer yringe was used for all titrations.

The Rothmund-Kornf ld equation is an empirical mod- ification of the Mass Action Law valid for weak cation exchangers (Samuelsson 1952). The equation implies that, when tw cations compete for the ionic sites of the ex changer plotting the log of the ratios between the con- centrations of the two ions in the xchanger vs the log of the corresponding concentrations in the suspension medium should give a straight line

$$\frac{[B_r]}{[A_r]} = \left(\frac{[A_s]}{[B_s]}\right)^{\beta} K$$

A_r and B_r ions in ion exchange resin. A_s and B_s = ions in solution β and K = empirical factors.

we described the ability of a dialyzed and thereby ATP free granule material from bovine adrenal medulla to bind inorganic and organic (biogenic amines) cations The binding could be ascribed to the cation exchanger properties of the material The similarity of the uptake curves the identical uptake maxima and the narrow pH range (between 4–7) for the uptake of all the inorganic and organic cations studied indicated the binding of these ions to common sites probably carboxyl groups

The fact that all the uptake curves fitted the Rothmund Kornfeld equation for weak cation exchangers corroborated the cation exchanger properties of the dialyzed material However the CA binding capacity corresponded to only 20–30 % of the normal in vivo content of CA in the bovine adrenal medulla.

In the present study we will describe the ability of medullary granule material to bind additional amounts of CA the total binding capacity in vitro being sufficient to comply with the storage requirements for CA in vivo Our observations indicate the existence—at least in vitro—of two storage pool The smaller of the two pools has the properties of a cation exchanger and binds unselectively inorganic and organic (e g biogenic amines) cations The other pool the large one retains selectively—probably stereospecifically—L NA and L A possibly by a combination of ionic and hydrogen bonds The two pools will in text and figures be denoted pool 1 and pool 2 respectively

METHODS

Preparation of granule material

Bovine adrenal medullary granules were isolated according to Poisner & Trifaró (1967) Adrenal were transported from the slaughter house on ice and the isolation procedure started about 3 h post mortem by homogenization of the medullas in ice-cold 0 3 M sucrose (glass homogenizator) After centrifugation at 800 g for 10 min the supernatant was sucked off and allowed to pass under slight N_2-pressure (700 kPa) through series of 5 mm millipore filters mounted in a Sartorious filter apparatus type SM 16253 The pore sizes consecutively used were 3 1.2 0 6 0 45 and 0 µm. The final filtrate was centrifuged at 20 000 × g for 10 min The opalescent supernatant was decanted together with the upper loose superficial sediment layer The walls of the tube and the pellet surface were carefully dried with filter paper In the electron microscope the remaining sediment was shown to consist of rather homogenous population of electron dense granules surrounded by a membrane (Fig 1)

The sediment was lysed in 10 ml of deionized water After careful stirring of the suspension 0 5 ml was removed for determining the dry weight and 100 µl for the CA content The rest of the suspension was used for studies on the uptake of inorganic and organic cations (and anions)

For some experiments the lysed material was dialysed against deionized water under continuous rocking for 4[illegible] (4 ml of suspension against 4000 ml of H_2O). All the procedures described above were performed in the cold room (~ +4°C)

Studies on the uptake storage and release of histamine and other biogenic amines in mast cell granules can simply be done on sedimental material from lysed granules since the amine binding granule material remains undissolved This is not the case with the matrix of medullary granules which to a great extent goes into solution on lysis of the granules. Only a minor binding capacity remains in the unsoluble precipitable rest (membranes etc) but is far too low to account for the original binding capacity of the granule material Since the CA binding material of the medullary granules was assumed to occur dissolved after lysis of the granules a technique was developed by which uptake studies could be performed on suspended lysed granule material which then was trapped under slight N_2 pressure (500 kPa) on filters selected to allow minor molecules like CA organic cations, ATP etc (molecules below ~10 000) to pass but to [illegible] proteins and other polymeres. When still moist the granule mass was scraped off and brought into pre weighed plastic tubes with stoppers. After determining the wet weight the samples were dried for 48 h over NaOH P_2O_5 in vacuo and the dry weight was determined. The dried material was then analysed for its uptake of biogenic amines, ATP inorganic cations etc as exemplified and described in detail below

Influence of pH on the uptake of L-NA

ATP (acid) was freshly prepared by passing a sodium-ATP salt (Na ATP 3.5 H_2O MW 614) through a 15 ml column of Dowex 50W–X2 (H^+) 5000 µmol of Na-ATP were put on the column and eluted with 25 ml of deionized water The content of ATP in the effluent was determined fluorimetrically (since a few per cent were lost during the ion exchange procedure) and the ATP concentration was then adjusted to 200 mM

10 000 µmol of L NA (base) was suspended in 5 ml of deionized H_2O containing 0 1 ml of H_2SO_4 [illegible]) The freshly prepared ATP (acid) conc. 200 mM was added under stirring until the L NA (base) had gone into solution At pH ~6 12 5 ml of the ATP solution corresponding to 2500 µmol were consumed to dissolve and neutralize 10 000 µmol of L NA (base)

0 25 ml of [14]C ATP (50 µl/ml) 0 25 ml of [3H]-L-NA ([illegible] µl/ml) and deionized H_2O to 25 ml were added Final pH 6 4

L NA–ATP salt solution was acidified to desired pH with conc HCl One ml samples were taken for uptake studies and 0 5 ml samples for the determination of the dry weight of the solutions (vacuum NaOH P_2O_5)

One ml of the lysed granule material was mixed [illegible] ml of the L NA ATP salt solution 25 µl of the mixture was pipetted into preweighed plastic tubes (11×55 mm) with stoppers The tubes were reweighed to get the weight

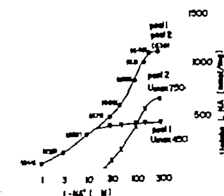


Fig. 4. Graphical construction of two-pool system for the storage of L-NA in dialyzed adrenal medullary granule material (from Fig. 3a). 1. ●—● total uptake of L-NA (phosphate). U_{max} 1200 nmol/mg. pH indicated by figures within brackets. ▼—▼ the initial uptake phase at the denoted pHs calculated according to the Rothmund Kornfeld equation. U_{max} 450 nmol/mg, pool 1. 3. — constructed second uptake phase by deduction of initial uptake curve from total uptake curve. U_{max} 750 nmol/mg = pool 2.

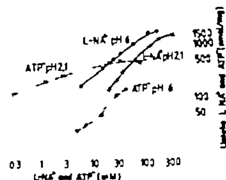


Fig. 5. Concentration dependent uptake of ATP^- and L-NA by lysed granule material at pH 5.1 and 6. ●—● L-NA pH 6. —— ATP^- pH 6. — L-NA pH 5.1. — ATP^- pH 5.1.

tion for a reconstruction of the L-NA uptake at pH 7.4 a typical uptake curve was obtained with a U_{max} of 450 nmol/mg (see Fig. 3a and compare with Fig. 7).

At L-NA concentrations above 10–30 mM the retention of L-NA by the granule material increased further reaching an apparent maximum around 1100–1200 nmol/mg at an L-NA concentration in the perfusion fluid ~300 mM. This second phase of L-NA uptake showed other characteristics than the initial one. It was quite resistent to acidification not being seriously reduced until below pH (for further discussion see page 350). The total uptake of L-NA varied between batches from 800 to 1200 nmol/mg dry material but was never observed to reach the values required for an uptake corresponding to the normal CA content of the adrenal medulla, ~2000 nmol/mg *net* dry weight (CA and ATP deducted) or ~1300 nmol/mg *total* granule dry weight, as reported by Hillarp (1956).

A graphical separation of the two phases of L-NA uptake is illustrated in Fig. 4. Using the Rothmund-Kornfeld equation the initial uptake phase could be completed for L-NA concentrations up to 300 mM and for the pH denoted on the uptake curve. The U_{max} obtained by this calculation was 450 nmol/mg. The deduction of the values thus obtained for the initial uptake phase from those for the total uptake yielded a curve with a U_{max} of 750 nmol/mg. The two uptake curves suggested to us the existence of two storage compartments, in Fig. 4 denoted pool 1 and pool 2, respectively.

Comment. Although the mechanism of storage of L-NA in pool remained to be studied the results obtained with the dialyzed material revealed the interesting fact that the retention of L-NA did not require ATP as anion or as a complexing agent. Fig. 3a and b show an uptake of L-NA both as phosphate and as chloride to about the same U_{max} ~1200 nmol/mg. To judge from the retention of admixed ^{32}PO (Fig. 3a) and ^{36}Cl (Fig. 3b) there was no corresponding uptake of these anions at the pHs used, 6.4–6.7 and 5.1–6.2, respectively.

Lysed—but not dialyzed—granule material

As evident from the above observations dialyzed granule material was able to retain considerable amounts of L-NA (800–1200 nmol/mg) although not as much as stored in the adrenal medulla in vivo (~ 000 nmol/mg CA, Hillarp 1959). Since dialysis might have reduced the CA binding capacity of the granule material by removing binding components or by causing conformational changes of the granule matrix etc. the subsequent studies were performed on lysed—but not dialyzed—material. Since such material contained ~6 µmol/ml of endogenous CA, and thereby pool 1 was expected to be more or less filled (see Figs and 3a) in most experiments no attempt were made to record separately the two uptake phases, only the total uptake being determined.

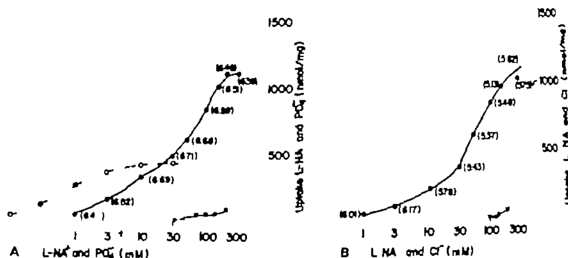


Fig 3a Concenetration dependent uptake of L NA and PO by dialyzed granule material Total uptake of L-NA (●—●) measured pHs within brackets Initial phase of L NA uptake (pool I) reconstructed according to the Rothmund Kornfeld equation for pH 7 4 (○—●) Total uptake of PO (■—■) For details see methods.

Fig 3b Concentration dependent uptake of L-NA and Cl by dialyzed granule material at 4°C Measured pHs within brackets Uptake of L NA (●—●) Uptake of Cl (■—■) For details see methods

MATERIALS

L-(7 H) noradrenaline hydrochloride (54 mCi/mg) DL-(7 H) adrenaline hydrochloride (53 mCi/mg) (side chain-1 2 H) tyramine hydrochloride (12 1 mCi/mg) (ethylamine 1,2 H) dopamine hydrochloride (~10 mCi/mmol) acetyl (N-methyl- H) choline chloride (1 1 mCi/mg) (ring C) histamine dihydrochloride (>50 mCi/mmol) (²²Na) sodium chloride in aqueous solution carrier free (>100 mCi/mg) (³⁶Cl) sodium chloride (>3 mCi/g Cl) (8- C) adenosine 5-triphosphate ammonium salt (61 mCi/mmol), (2 H) adenosine 5-triphosphate ammonium salt (1 500–30 000 mCi/mmol) were obtained from the Radiochemical Centre Amersham

(Ethyl-1 C) β-phenyl ethylamine hydrochloride (333 μCi/mg) (³²P) disodium phosphate (50–1 000 mCi/mmol) were obtained from New England Nuclear Boston

ABBREVIATIONS

A = adrenaline NA = noradrenaline TA = tyramine PhEA = phenylethylamine DA = dopamine CA = catecholamine ATP = adenosine-5 triphosphate Hi = histamine Ach = acetylcholine Na = sodium

RESULTS

I *Dialyzed granule material*

Since our previous studies on the binding of biogenic amines (Uvnäs & Åborg 1977) were performed on dialyzed granule material thus depleted not only of CA but also of ATP and inorganic ions our initial experiments in the present series were performed on such material

Confirming our previous observations the dialyzed granule material was found to retain e g L NA and Na At pH ~7 and within a concentration range up to 10–30 mM in the perfusion fluid the uptake of these cations approached a U_{max} of 400-500 nmol/mg dried granule material (Fig. ?) and the uptake curves fitted the Rothmund Kornfeld equation for weak cation exchangers as previously described by Uvnas & Åborg (1977) However when the L NA concentrations were increased above 10–30 mM the L NA uptake curve was observed to bend more or less steeply upwards indicating the appearance of a second phase of uptake In the initial experiments which were performed as close to neutral pH as possible the uptake of L NA could reach unreasonably high values without distinguishable maxima Since NA has a low solubility and is reported to have a tendency to aggregate at neutral pH we tried to avoid these complications by lowering the pH of the perfusion fluid

Fig 3*a* and 3*b* illustrate two uptake curves, one for L NA phosphate (3*a*) and one for L-NA hydrochloride (3*b*) both experiments performed at slightly acid pHs as indicated in the figures.

As could be foreseen from our previous observations (Uvnas & Åborg 1c) there was a considerable distortion and shift to the right of the uptake curve at L NA concentrations <30 mM due to the high sensitivity of the initial phase of uptake to acidification However using the Rot und Kornfeld equa-

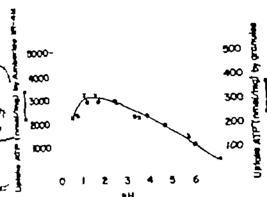


Fig. 9 Influence of pH on uptake of ATP by Amberlite IR-4B (anionic exchanger) (—) and by lysed granule material (●—●). Concentration ATP⁻ 50 mM.

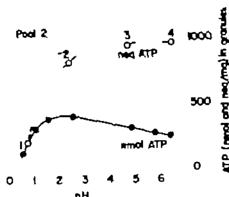


Fig. 10 Influence of pH on the uptake of ATP by lysed granule material with L NA as the cation. L NA 200 mM, ATP 50 mM. Uptake ATP in nmoles (●—●) in neq (○——○). The valencies of ATP obtained by titration with HCl denoted by Figs. 1, 2, 3 and 4 respectively.

common biogenic amines tested (PhEA TA DA NA, A, HI, Ach) that were retained above 500 nmol/mg, the U_{max} of pool 1 (Fig. 7). The uptake of L NA and DL A into pool was ~900 and ~500 nmol/mg, respectively.

(b) Uptake of sodium ion

Sodium ions showed a pH sensitive uptake with a U_{max} around 500 nmol/mg (Figs. 6 and 8), in other words, an uptake corresponding to the cation exchanger pool (pool 1). Sodium ions were unable to penetrate into pool 2 even when concentrations of 100-300 mM were used (Figs. 6, 7 and 8).

(c) Uptake of ATP and other anions

There was no correlation between the retention of L NA and the anions PO and Cl⁻ (Figs. 3, 3b and 5), either when concentrations or pH were varied. When offered as sodium salts the uptake of ATP⁻ PO⁻ and Cl (in Fig. 8 shown for ATP) increased with increasing acidity indicating the appearance of cationic binding sites—presumably NH_3^+ groups. The conformity between the uptake curves for sodium–ATP by the granule material and by an anion exchanger (IR-4 B) is obvious (Fig. 9). However, when perfused through the granule material as NA ATP salt, ATP was retained equivalently to NA (Fig. 10), an important phenomenon which will be discussed on page 353.

The possibility that this equivalent uptake of NA and ATP was due to the occurrence of these ions as a complex could be excluded by the fact that the titration curves for Na_4 ATP and NA_4 ATP (at 50 mM conc. pH<1 up to 6.5) with HCl were identical.

DISCUSSION

Adrenal medullary granules were lysed by deionized water to disrupt the granule membranes. When such material—with or without previous dialysis—was perfused under slight pressure (10 psi) at 4°C with solutions containing organic (biogenic amines) or inorganic cations these were retained.

The biogenic amines were distributed within two pools. One of the pools (denoted pool 1) was fully charged at amine concentrations >10 mM up to U_{max} of 400–500 nmol/mg. Pool 1 accepted unselectively biogenic amines (PhEA TA DA NA A and HI) and inorganic cations (N and Ca^{2+}) and had the characteristics of a cation exchanger. The other pool (pool) was selective for NA and A, the only amines among the above mentioned which accumulated towards a higher U_{max} (around 1500–000 nmol/mg for L NA).

The presence of ATP has been considered essential for the storage of CA in the adrenal medulla either for ionic binding of CA (Blaschko et al. 1976, Falck et al. 1956) or as component in aggregate formation (Berneis et al. 1971), possibly together with chelating ions (Ca^{2+} and Mg^{2+}). The present observations show that dialyzed granule material depleted of these components still has a considerable ability to retain L NA. Lysed but not dialyzed material has still greater ability to

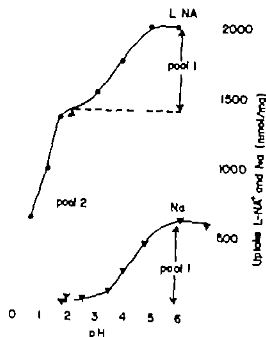


Fig 6 Influence of pH on uptake of L NA and of Na by lysed granule material Concentration of L NA,ATP and NaCl 200 mM Note L NA enters both pool I and Na only pool I For details see methods

(a) Uptake of CA and other biogenic amines

Concentration dependence The non-dialyzed material had a considerably greater ability to retain L-NA than the dialyzed material Within a concentration range of 10–300 mM L NA *in the perfusate* a concentration dependent uptake occurred The maximal uptake capacity varied somewhat between

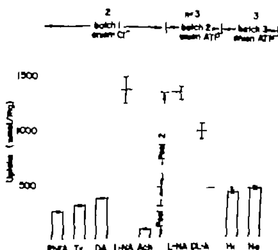


Fig 7 Total uptake by lysed granule material of various biogenic amines and of Na ions. Concentration throughout 100 mM Note Only L NA and DL A penetrate into pool 2 Uptakes of NA as phosphate and as ATP salt are equal

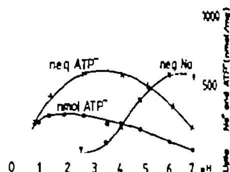


Fig 8 Influence of pH on uptake of ATP and Na by lysed granule material Concentration of ATP 50 mM and of Na 193.5 mM

batches usually being between 1500–2000 nmol/mg (Figs 5 6 and 7)

pH Dependence In contrast to the initial uptake of L NA (pool I) the second phase of uptake was rather independent of pH It is true that at pH 2.1 the uptake curve for L NA was shifted somewhat to the right (Fig 5) but the maximal uptake was still ~1600 nmol/mg The difference between the maximal uptakes at pH ~6 and 2.1 was 500 nmol/mg This figure corresponds fairly well with the size of pool I which is abolished at pH 2.

The influence of pH on the uptake of L NA is seen more in detail in Fig 6 The uptake curve shows an inflexion point at pH ~2 Above this point the course of the curve reflects the pH sensitivity of the L NA uptake into pool I It should be compared with the corresponding sodium curve below in the same figure Sodium ions enter only pool I as discussed below and shown in Fig 8. The deduction of pool I (~600 nmol/mg for both L-NA and Na) from the total uptake yields pool 2 (~1400 nmol/mg) which as already shown in Fig 3 is independent of pH above ~2

Influence of anions The uptake of L NA was independent of the accompanying anion as demonstrated for ATP Cl and PO in Figs 3a 3b and 7 In the presence of any of these anions the U_{max} for the total uptake reached values around 1500–2000 nmol/mg

Specific uptake of L NA and DL A As demonstrated previously (Uvnäs & Åborg 1977) pool I (the cation exchanger pool) unselectively retains inorganic and organic (biogenic amines) cations with a U_{max} ~400–500 nmol/mg granule material In contrast pool 2 showed to be specific for L NA and DL A which were the only two amines of the 7

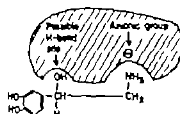


Fig. 12. Hypothetical model of ATP-independent binding of L-NA and L-A in adrenal medullary chromaffin granules

ring are not essential for the binding mechanism. Presumably then the hydroxyl group in the side chain of NA and A is the essential one behind the specific binding of these amines. The mode of binding of the NA and A can only be speculated upon. To account for the rather pH insensitive but reversible binding of the strongly charged amines one might imagine binding pocket with an anionic site for the charged amino group—e.g. a COO⁻ group—and a hydrogen bond site for the OH group at the asymmetric carbon atom of the ethyl amine chain (Fig. 12).

We believe that the strongly charged chromogranine molecules—as was shown for the heparin-protamine salt complex—are likely to contain COO⁻— NH salt linkages forceable by NA and A ions but inaccessible to inorganic cations e.g. Na ions. As pointed out above the ability of NA and A to force the storage pocket of pool is independent of ATP. Although e.g. Cl⁻ and PO⁻ ions do not enter as indicated in Figs. 2a–3b when NA was offered the granule material as NA ATP salt ATP was accepted equivalently with NA. The likely explanation is a binding of the acid ATP to ⁺NH₃ groups which become demasked when NA ions break COO⁻ — NH salt bridges and which have a affinity to the phosphate groups of ATP (Fig. 1). Whether in addition to the binding of CA to COO⁻ groups and of ATP⁻ to NH groups also electrostatic interaction occurs between CA and ATP can only be speculated upon. Anyhow this postulated equivalent binding of CA and ATP within one and the same "binding pocket" which behaves like combined anion–cation exchanger should explain the concomitant release of CA and ATP reported by so many authors. The closing of the "storage pocket" after the two storage components should ensure desired electro-neutrality in the storage machinery.

Unfortunately we had only labelled L-NA and DL-A at our disposal and could therefore not directly study the possible stereospecificity of the CA uptake. However with the same concentrations of the two amines in the perfusate (100 mM) the amount of DL A retained in pool were ~55% of the amounts of L NA retained (Fig. 7). Since the two amines seem to have the same affinity to the binding sites of pool (to be published) the observation is compatible with a stereospecific binding of both L NA and L A.

The present observations were performed on lysed granules at 4°C in order to minimize the uncontrollable influences of active membrane transport processes as well as of enzyme activities. Thus the observed uptake storage and release phenomena can be characterized as physico-chemical processes non-dependent on metabolic energy.

The functional implication of a granule two-pool storage of NA and A of the kind described above will be discussed in a forth-coming paper in which a cation dependent release of the two CA from medullary granules in vitro is described.

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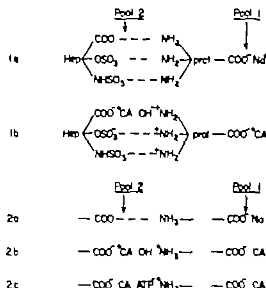


Fig 11 Schematic drawings illustrating the presumed characters of pool 1 (the cation exchanger pool) and of pool (the storage pool) (1) in a heparin–protamine complex (Uvnas & Åborg 1976) ( ) in lysed medullary granule material When exposed to sodium ions these ions become attached to free COO groups but are unable to break COO -- NH (NH) salt bridges (1*a* and *a*) When exposed to CA (as hydrochloride or phosphate) CA ions primarily become attached too free COO groups but in higher concentrations CA ions force COO — NH (NH) salt bridges open and become attached to the thereby exposed COO groups (1*b* and 2*b*) Both pool 1 and pool become carhged with CA but the anions Cl do not enter When exposed to CA as ATP salt also the ATP enters pool presumably in ionic linkage with exposed NH groups (*c*)

store NA corresponding to the normal amounts of CA in the adrenal medulla in vivo

The mechanism of the NA storage in pool ? can only be speculated upon It is evidently independent of ATP since the uptake (Fig 7) and the storage capacity as reflected in the U_{max} (Fig 3*a* and *b*) are as high with Cl and PO as with ATP In contrast to pool 1 pool ? is rather independent of pH While the storage ability of pool 1 rapidly deteriorates with increasing acidity and i virtually abolished at pH 3 pool ? resist acidification down towards pH ? before it begins to diminish The concentrations of NA and A required for forcing pool ? are rather high—above 10–30 mM and U_{max} requires concentrations ~?00–300 mM However it should be remembered that these concentrations of CA are comparable to those calculated to exist in the medullary granules in vivo (Hillarp 1959)

In its demands for high CA concentrations its resistance against acidification its reversibility—the storage of CA increasing with raising and decreasing with falling external CA concentrations—and its inaccessibility to sodium ions pool ? of the medullary granule material reminds of the second storage pool of a heparin–protamine complex described by Uvnäs & Åborg 1976 (Fig 11). This salt complex had one cation exchanger pool accessible to both biogenic amines and inorganic cations with the terminal protamine carboxyl groups as the cation binding sites (pool 1) and a second pool (pool 2) accessible to aromatic biogenic amines rather insensitive to acidification and unavailable to inorganic cations like sodium ions For the opening of pool ? of the heparin–protamine complex relatively high concentrations of the amines were required (>10–30 mM) From calculations on the binding capacity of the two pools of the complex was concluded that when pool 1 was filled higher concentrations of the amines were able to force open the salt bridge between heparin and protamine, primarily the weaker COO H_3N bonds and secondly (with even higher concentrations) also the stronger OSO – H_3N bonds The attachment of the amines to the thereby demasked binding sites was reversible and the salt bridges closed as soon as the external amine concentrations were reduced Only a minor part of the amines were retained in spite of lowering the external amine concentrations, probably due to attachment to the stronger SO_3 groups

The medullary granule material has no heparin to form a complex similar to that of the mast cell granules The proportions of chondroitin sulphate and other sulfomucopolysaccharides are also too small to form protein complexes of sufficient storing capacity (DaPrada et al 197?) However the proteins which form the matrix of the adrenal medullary granules are considerably acid with presumably many end terminal α-carboxyls, and β- and γ-carboxyls from aspartic and glutamic acid respectively Titration of granule material for free COO groups (figures not shown) revealed more than enough of such groups (~1 ?00 nmol/mg) than required (500–600 nmol/mg) for binding of the CA retained in pool 1 the exchanger pool but not sufficient for the CA binding in pool

The binding of NA and A in pool 2 is specific, the other aromatic amines tested—PhEA TA and DA or heterogenic amines like Hi and Ach not being able to force this pool open Especially notable is the inability of the catecholamine DA to do so, indicating that the OH-groups of the catecholamine

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In vitro studies on a cation dependent catecholamine release from a two-compartment storage in bovine adrenal medullary granules

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UVNÄS, B. & ÅBORG, C. H. In vitro studies on a cation dependent catecholamine release from a two-compartment storage in bovine adrenal medullary granules. Acta Physiol Scand 1980, 109: 355–362. Received 21 Nov. 1979. ISSN 0001-6772. Department of Pharmacology, Karolinska Institutet, Stockholm, Sweden.

Perfusion of adrenal medullary granules, isolated according to Posner & Trifaró, with isotonic cation-containing solutions (NaCl, KCl, LaCl) caused a concentration dependent release of CA. The course of the release indicated the existence of a two-compartment storage of CA. The minor compartment (pool 1) with a U_{max} of ~500 nmol/mg granule dry weight showed the characteristics of a cation exchanger and was assumed to be depleted on exposure to cations, e.g. sodium ions, as an ion exchange $Na^+ \rightleftharpoons CA$. The major compartment (pool 2) with a U_{max} of ~1000 nmol/mg stored both CA and ATP. It was assumed to empty via the cation exchanger pool. The total storage capacity of the two compartments ~1500 nmol/mg matched the in vivo storage capacity for CA (~1300 nmol/mg according to Hillarp 1960).

Key words: Adrenal medullary granules, storage of catecholamines, noradrenaline, adrenaline, adrenal medulla

In two preceding papers (Uvnäs & Åborg 1977, 1980) we have described the ability of lysed granule material from bovine adrenal medulla to retain or take up biogenic amines) and inorganic cations. The observations indicated the existence of two storage compartments. One of these, denoted pool 1, showed the characteristics of a cation exchanger storing unselectively biogenic amines and inorganic cations. The storing capacity of pool 1 amounted to ~400–500 nmol/mg dried granule material. The other compartment, denoted pool 2, showed a selectivity for NA and A, with a storage capacity for these CA ~1000–1500 nmol/mg granule material. The binding of CA in this pool was tentatively ascribed to the attachment of the amino and hydroxyl groups of the amine side chain to anionic and hydrogen bond sites in the granule matrix (Uvnäs & Åborg 1980). Altogether the total storage capacity for NA and A in the lysed granule material sufficed for the storage of the normally occurring content of the catecholamines (~1300 nmol/mg total granule dry-weight according to Falck et al. 1956).

Retained ATP was assumed to be linked—equivalently with CA—to ionized NH groups, unmasked on the opening by CA of hypothetical COO⁻ --- H_3N salt bridges.

In this paper will be described a cation dependent release of NA and A from a two-pool storage system in medullary granules in vitro, with an attempt to explain the functional interplay between the two pools.

METHODS

Bovine adrenal medullary granules were prepared by millipore filtration of homogenized adrenal medullae according to Posner & Trifaró (1967), as described by Uvnäs & Åborg (1980). The granules were resuspended in 0.32 M sucrose and 1 ml of this suspension put on a 25 mm Ø filter, pore size 0.01 µM Sartorius SM 11311 in a Sartorius filter apparatus Type SM 16223. The sucrose solution was removed under an N_2 gas pressure of 700 KPa. A second filter of the same characteristics as above, moistened in sucrose was then placed over the granule layer and tightened with a rubber packing.

7 ml of isotonic NaCl or other isotonic solutions (usually buffered with 10% Na_2HPO_4 + H_2SO_4 to pH 7.5) were pressed through under an N_2 pressure of 700 KPa. The effluent were collected in preweighed plastic tubes (5.5×1.1 cm) with stoppers in 0.25–0.5 ml samples. The

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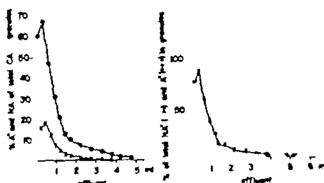


Fig 3 (a) & (b) Concomitant release of A and NA from granules perfused with isotonic (163 mM) NaCl expressed as (a) per cent of total CA, (b) per cent A and NA of total A and NA respectively

against the bound CA according to Scatchard (Fig 5c). The plot may suggest a two-pool system with a maximal storage capacity of ~500 and ~1000 nmol/mg respectively. A reciprocal plotting of the CA concentrations in the perfusion fluid vs. the corresponding concentrations in the granules may likewise be interpreted as indicating a two-pool system with maximal capacity values around 500 and 1000 nmol/mg respectively (Fig. 5d). Plotting the effluent values according to the Rothmund-Kornfeld equation for weak cation exchangers showed that only the values corresponding to the latter part of the effluent curve—points 9–15—fit this equation while the earlier effluent values do not (Fig 5e).

The curves plotted above may be taken to suggest that the granule CA store comprises compartments, one the minor one with the characteristics of a weak cation exchanger, the other the larger one of so far undefined nature.

An attempt to envisage the interplay between the two hypothetical CA storage pools instrumental at sodium induced CA release is seen in Fig. 6. Using values from curves 5a–5 the curves in Fig. 6 were constructed. By deduction of the curve for pool 1 (the exchanger pool) from the curve for the total store the curve for pool is visualized. Fig. 6 also illustrates the inverse relationship between the CA release and the Na uptake by ion exchange in pool 1 with the constant sum of intragranular CA and Na as demonstrated above (Fig 4). The curves will be further commented upon under Discussion p 7

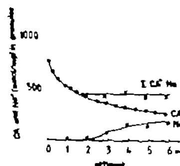


Fig 4 Release of CA and uptake of Na in granules perfused with isotonic (163 mM) NaCl buffered with 10% sodium phosphate buffer to pH 7.4. Release of CA (O—O); uptake of Na (●—●). Sum of CA release and Na uptake by the granules (—). Note Σ CA and Na constant at ~500 nmol/mg corresponding to the U_{max} of pool 1 the cation exchanger pool.

NA induced inhibition of CA release

The slight leakage of CA observed on perfusion of the granules with isotonic sucrose was inhibited by the addition of L NA to the perfusion fluid. In the presence of 10 mM L NA the net loss of CA levelled off at an intragranule concentration ~1050 nmol/mg (Fig. 7). Noteworthy is the agreement between this experimentally obtained value and the corresponding value that could be predicted from Fig. 6 (point a). The presence of 10 mM L-NA also limited the CA released induced by perfusion of the granules with isotonic sodium chloride solution. In fact the net release of CA was arrested at an intragranule concentration ~550 nmol/mg. Also this value is pretty close to the one predictable from Fig. 6 (point b) at this extragranule CA concentration

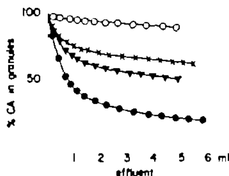


Fig 1 Release of CA from granule perfused with isotonic solutions containing deionized sucrose (O—O) sucrose +40 mM Na (×—×) sucrose +80 mM Na (▼—▼) and 163 mM Na (●—●) All the NaCl containing solutions buffered to pH 7 4 with 10% sodium phosphate buffer

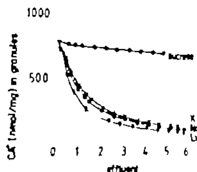


Fig 2 Release of CA from granules perfused with isotonic solution of sucrose (O—O), potassium chloride (×—×) sodium chloride (●—●) and lithium chloride (▼—▼) All cation containing solutions buffered to pH 7 4 with phosphate buffer of the respective cation

exact volumes were obtained by weighing 25 µl from each sample were mixed with 5 ml 0 1 M HCl containing 0 0025 M H_2SO_4 CA was determined spectrofluorimetrically (fluorescence 325 nm excitation 285 nm)

0 1 ml from each sample was used for protein determination according to Lowry et al (1951)

ABBREVIATIONS

CA=catecholamine A=adrenaline NA=noradrenaline ATP=adenosine 5 triphosphate

RESULTS

Cation induced CA release

Perfusion with isotonic deionized and unbuffered sucrose solution resulted in a slight release of CA (Fig 1) However when the sucrose was exchanged for sodium chloride the release was accelerated and enhanced the more so the higher the sodium content (Fig 1) Isotonic solutions of K Na and Li-chloride yielded very similar release curves (Fig 2) The concomitant outflow of PCA precipitable protein amounted to less than % of the total granule content of such protein indicating negligeable losses of granule matrix protein

The release included both A and NA with practically identical release curves for the two amines on exposure of the granules to isotonic NaCl solution (Fig. 3*a* and 3*b*)

Entrance of sodium ions into granules

Concomitant recordings were made of the appearance of CA in the perfusion fluid and the entrance of Na ions into the granules The granules were perfused with isotonic NaCl solution No detectable accumulation of sodium (^{22}Na) occurred in the granules until their CA content had declined below 500 nmol/mg From then on a continuous accumulation of Na occurred concomitantly with a continued depletion of the CA content (Fig 4). Noticeable is the fact that the sum of the equivalents of Na which entered the granules and those of the CA that left the granules remained constant at ~500 nmol/mg It is further noticeable that the entrance of Na into the granules began first when the intragranular content of CA had fallen to ~500 nmol/mg just the CA level which according to the calculations below (Fig. 5) represents the U_{max} of pool 1 the cation exchanger pool The observations thus agree with an ion exchange between Na and CA ion in pool 1

Evidence for a two-compartment storage of CA

Our previous studies on the CA storing ability of lysed granule material had suggested the existence of a two-pool storage of CA in that material An analysis of the release courses for CA from perfused granules induced by the cations Na K and Li suggested a similar two-pool system in the intragranule storage of CA The release curves in Figs 5*a* and 5*b* show the release of CA (µmol/ml) into the effluent and the remaining CA (nmol/mg) in the granules respectively on perfusion of the granules with 163 mM NaCl Out of totally 1097 nmol/mg in the granules 9 4 nmol/mg were released during the perfusion

Assuming some kind of competitive binding of CA to storage sites in the two pools the ratio between granule bound and CA was plotted

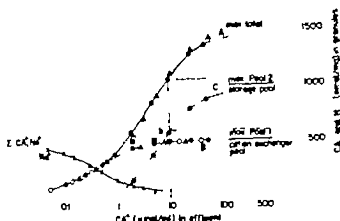


Fig. 8 Constructed curv to illustrate the assumed interaction within two-pool system for the storage and release of CA in adrenal medullary granules Curv A Total CA store ▲—▲ from Scatchard plot (Fig 5); ●—● from Lineweaver-Burk plot (Fig 5d). Curv B CA in pool 1 the xchanger pool. ○—○ from Rothmund-Kornf ld (Fig 5e); ▲—▲ from Scatchard plot (Fig 5c) ●—● from Lineweaver-Burk plot (Fig 5d) Curv C CA in pool ... storage pool. Curv B (xchanger pool) deduced from Curve A (total CA store). — Sodium content in pool 1 (from Rothmund Kornfeld) (Fig 5e). ■—■ Σ CA NA in pool 1

from intact granules yield picture of a two-pool storage system very similar to that one obtained from our previous uptake studies on lysed granule material (Uvnäs & Åborg 1980, Fig. 3) The intragranule localization, the chemical construction and the inter and intramolecular arrangement of the two pools are unknown, but an ttempt to interprete our observations in functional terms and to en is age a possible interplay between the pool is schematically indicated in Fig. 8 This picture should not be seen as an indication of the patial arrangement of the two pool only as an att pt to visualize the role of pool 1 a transfer p d for the release of pool ..., the storage pool in our hypothetical scheme However the two pools seem to be localized to the esicle matrix mce no appreciable CA toring capacity was observed in the granule membrane fraction precipitated by d fferential centrifugation (100 000× g, 30 min) The storing capacity rema ned in the supernatant

According to ou hypothesis the one of the CA containing pool by us denoted pool 1 has the properties of a cation exchanger When this pool is exposed to biogenic am nes (in o presumably mainly A and NA) or inorganic cation (in vivo presumably mainly sodium ions) these positively charged ions will be unselectively retained according to the prevailing concentrations and to their affin ties to the anionic binding sites in the pool, probably protein carboxyls The storage ability of pool 1 is rather sensitive to acidification (see e g. Fig 5 in Uvnäs & Åborg 1980) and might therefore depend on the β and γ carboxyls of aspartic and glutamic acids which have pK values ~3 7 and 4.3 respectively Pool 1 has maximal storage capacity ~500 nmol/mg granule dry weight at external cation concentration ~10 mM at neutral pH

The other of the two CA containing pools by denoted pool 2, is in contrast to pool 1 specific—to judge from our observations on lysed granule ma-

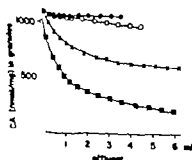


Fig 7 Inhibitory effect of 10 mM NA (anion ATP) on CA release Granules perfused with isotonic sucrose (○—○) isotonic sucrose 10 mM NA (●—●); isotonic (163 mM) NaCl (■—■), isotonic NaCl 10 mM NA (anion ATP) (×—), pH ~6 2

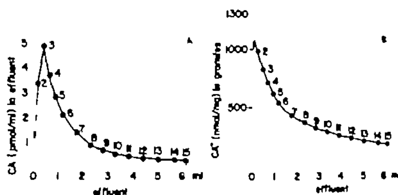


Fig 5a Release of CA (µmol/ml effluent) on perfusion of granules with 163 mM NaCl buffered with 10% sodium phosphate buffer to 7 4

Fig 5b CA (nmol/mg) in granules perfused with isotonic (163 mM NaCl) buffered with 10% sodium phosphate buffer to pH 7 4

DISCUSSION

A two-compartment storage of medullary CA

Supported by the present studies on the release of CA from isolated granules and the previous studies on the uptake of CA by lysed granule material (Uvnäs & Åborg 1979) we propose a two-pool storage of CA in the adrenal medullary granules. Fig 6 shows a graphical construction of these two pools based on the cation induced CA release observed in the present studies. These values for the CA release

Fig 5c Scatchard plot derived from the release curves in Fig 5a and 5b

Fig 5d Lineweaver Burk plot derived from Fig 5a and 5b

Fig 5e Logarithm plotting of the ratios between the concentration of CA and Na in the granules vs. the corresponding concentrations in the effluent on perfusion of the granule with 163 mM NaCl buffered to pH 7 4 with 10% sodium phosphate buffer. Values obtained from Fig 5a and 5b. The values follow a straight line i e fulfil the Rothmund-Kornfeld equation for a weak cation exchanger. U_{max} 500 nmol/mg

When medullary granules are perfused with inorganic cations, i.e. sodium ions, such ions will penetrate into the granules. Na ions are unable to enter pool ... hich is specific for L A and L-NA (as shown previously Uvnäs & Åborg 1979) but will compete ith CA ions at the ionic sites of pool 1. A ions are released and pass out through the granule membrane (arrow 1 and 2 in Fig. 8). However as long as pool is filled pool 1 will be under a permanent high CA ionic pressure the more so the more pool is filled. The Na ions which enter pool ll therefore immediately be replaced by CA ons from pool (arrows 3 and 4 in Fig. 8). If completely filled the CA ionic pressure will correspond to a CA concentration of 200–300 mM. This concentration more than 10-fold exceeds the A concentration required for the saturation of pool 1 (10 mM). No detectable amounts of Na ions ll therefore be able to accumulate in pool 1. However as pool is depleted the CA ionic pressure on pool 1 declines, Na ions can more favourably compete for the ionic sites of pool 1 and the CA release is increasingly accompanied by the accumulation of Na ions in this pool as illustrated in Fig. 6. The concentrations of NaCl required to release CA might seem high but are explained by the fact that the affinity of CA ions for the ionic sites of the ion exchanger pool is >10 times higher than the affinity of Na ions for the same sites (Uvnäs & Åborg 1977).

The idea of two-compartment store of CA in the adrenal medulla is not new. Hillarp (1960) suggested the existence of two storage pools to explain the observed equivalent excess of CA over ATP. Similar arguments for a two-pool system were brought forward by Stjärne (1964), Taugner & Hasselbach (1966) and others.

In the present two-pool hypothesis the larger compartment (pool 2) should correspond to the protein-ATP-CA complex postulated by various authors. Even if according to our observations ATP is not essential for the binding of CA in pool as demonstrated on lysed granule material it might serve as a stabilizing factor by its possible electrostatic attraction to CA (Fig. 8). The indicated intermolecular arrangement between CA and ATP should also explain the concomitant release of CA and ATP. When the CA ions move from pool 2 to pool 1 the neutrality is maintained by the simultaneous loosening of ATP⁻. The COO and H_3N groups of the storage sites reattach to each other and neutrality becomes restored by the closing of the "storage pocket".

The smaller compartment pool 1—in our scheme the cation exchanger pool—should correspond to the previously suggested ATP-free CA pool.

The existence of a third pool of free diffusible CA has been lively discussed (Euler 1956, Stjärne 1964). In fact in order to uphold the ionic balance and the ion flux between pool 1 and pool in our scheme as well as between pool 1 (the release pool) and the extragranule space minute fractions of diffusible or free CA ions have to be imagined. Whether these diffusible minute fractions of CA ions should be denoted pools or not seems to us to be more of a semantic question.

In this context the observed leakage of CA from granules perfused with isotonic sucrose might deserve some comments. Since the granule membranes at 0°C are permeable to CA ions a leakage of CA will be an inevitable consequence of the existence of free CA pools as discussed above. This observed leakage of CA seemed to be contradictory to previous reports that granules suspended in isotonic media maintained their CA content for days (Falck et al. 1956 and others). However we found that iterated resuspensions of batches of granules in fresh isotonic sucrose or salt solution led to a tepwise reduction of the granule CA content. After each resuspension the CA content in the granules assumed a new lower level but remained then constant for hours indicating that the retention of CA by the granules depended on the equilibrium formed between intragranule and in the suspension fluid accumulated CA. In our perfusion system such an equilibrium between i tra- and extragranule CA concentrations will not occur due to *the continuou removal of the released CA*. However as predicted from the above reasoning the net loss of CA from our perfused granules could be effectively counteracted by adding NA in appropriate concentrations to the perfusion fluid. In other words, the observed ability of isolated medullary granules to retain CA at 4°C is not due to any unique properties of the granule membrane. It is the simple result of a physico-chemical balance between intra- and xtragranule CA.

The experiments described in the present article have all been performed on isolated medullary granules at 4°C. Consequently membrane transport processes have been "paralysed" and enzymatic processes practically abolished. The observed in-

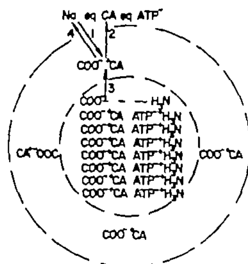


Fig 8 Schematic drawing of the functional interplay between cation exchanger pool (pool 1) and storage pool (pool 2) For explanation see text p ■■ The picture should not be taken to suggest the spatial organisation of the two pools in the granule

terial possibly stereospecific—for L A and L NA The maximal storage capacity of pool is ~1000 nmol/mg For its filling pool 2 requires higher external CA concentrations than pool 1 for maximal uptake ~200–300 mM Pool 2 is reversible the number of L A and L NA storing "pockets increasing with raising and decreasing with falling CA concentrations Since as already mentioned above pool 2 is specific for L A and L NA (DA is not retained in this pool only in the unselective pool 1) and the hydroxyl group in the catecholamine side chain seems to be essential for the storage in pool 2 we have suggested (Uvnäs & Åborg 1979 Fig 9) that L A and L NA are held in pool 2 by the combination of ionic binding of the NH_3 groups in the amine side chain to protein carboxyls and of hydrogen bonds between the hydroxyl group of the side chain and hydrogen binding sites of the granule protein Since pool 2 is rather insensitive to acidification—the storage mechanism remains seemingly intact down towards pH ~ —terminal protein carboxyls with their pK a ~2 might be involved as binding sites

The sum of the storage capacity of pool 1 (~500 nmol/mg) and pool 2 (~1000 nmol/mg) compares well with the value for the total content of CA in the bovine adrenal medulla (~1300 nmol/mg) reported by Hillarp et al 1955

In the present experiments ATP was observed to be released from the granules concomitantly and equivalently with CA (unpublished) This observation is in accordance with a storage of ATP in pool 2 as proposed by Uvnäs & Åborg 1979 and schematically illustrated in Fig. 8. According to their proposal ATP is ionically linked to NH_3 groups which become unmasked on the opening by CA of hypothetical COO --- NH salt bridges in the protein matrix of the chromaffin granules CA is ionically linked to COO groups in the same storage pocket This simple mechanism of CA and ATP binding to ionic sites of opposite charge in a reversible storage pocket explains the equivalent storage and release of the two components as so often reported in the literature By this reversible binding mechanism the desirable electroneutrality of the storage mechanism is automatically secured. Additional stabilizing intermolecular attraction forces between CA and ATP as suggested by many authors to form an important part of the storage mechanism is easily compatible with our storage hypothesis However as pointed out in our previous paper (Uvnäs & Åborg l c) the presence of ATP is not a prerequisite for the storage of CA. Only A and NA are able to force the specific storage sites of pool 2 and ATP⁻ will enter as ion of opposite charge according to supply Since Cl and PO ions do not enter the storage sites these sites will probably in the lack of ATP be supplied with OH ions Excess of ATP on the other hand will not lead to an increased storage of ATP in pool 2 and as shown by studies with sodium-ATP ATP ions alone are not able to enter pool

Functional interplay between pool 1 and pool 2

As well known at 0°C the membrane of isolated medullary granules is permeable to smaller ions like CA ATP Na etc but impermeable to macromolecules (for ref see Stjärne 1975) We would therefore like to see the release of CA occurring in the present experiments on perfusion of the medullary granules with isotonic NaCl solution as the result of a cation exchange between CA and Na ions in pool 1 the exchanger pool Such an interpretation might at first sight seem incompatible with the fact that no accumulation of Na ions is observed to occur in the granules until they have lost a major part of their CA However guided by Figs and 8 we want to envisage the possible interplay between the two CA pools with a cation exchange over pool 1 as the essential step Our working hypothesis is as follows

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Possible role of nerve impulse induced sodium ion flux in a proposed multivesicular fractional release of adrenaline and noradrenaline from the chromaffin cell

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Based on own observations concerning a two-compartment storage of CA in the adrenal medulla and cation exchange dependent release of CA from perfused chromaffin granules in vitro and encouraged by recent reports from other laboratories about the importance of sodium ions for the CA release from the adrenal gland we propose a modification of the current quantal theory of CA secretion. Instead of secretion of quanta each quantum corresponding to the content of one vesicle we envisage a concomitant fractional release of CA from multiple vesicles adjacent to the chromaffin cell membrane. The CA secretion should be the result of cation exchange across the contact area between the plasma membrane and the granule membrane during the period of depolarization caused by the nerve impulse. The size of the released quanta should be determined by the nerve impulse induced sodium ion flux and the number of such ions which reach the CA binding ionic sites in the cation exchanger pool (the release pool) of the granules

Key words Adrenaline noradrenaline release chromaffin cell adrenal medulla

According to the doctrine of the day the secretion of the adrenal catecholamines (CA) is the result of an exocytosis of CA containing vesicles with a total evacuation of the affected vesicles not only of their CA but also concomitantly of all other soluble components like ATP chromogranines dopamine β-hydroxylase etc This all or none exocytotic response implies that the CA secretion is quantal with the content of a vesicle as the smallest quantum to be secreted The vesicles are assumed to be in constant motion (Brownian motion) and to collide randomly with the plasma membrane of the chromaffin cell. On nervous and humoral stimulation active sites are assumed to occur in the chromaffin cell membrane and vesicles which hit such an activated site become attached to the plasma membrane. The granule and plasma membranes fuse and an opening is formed through which the vesicle content is evacuated into the extracellular space (Fig. 1 and 1*b*)

The quantal theory with the CA content of one vesicle as the smallest releasable quantum further implies that this quantum is the smallest amount to be released by a nerve impulse A chromaffin cell from cat adrenal is claimed to contain 13 000 CA vesicles (Kirshner & Viveros 1970) The amount of CA released by one splanchnic nerve stimulus is calculated to 3.5×10 (Celander 1954) and to 3×10 (Kirshner & Viveros 1970) of the total CA content of the gland This amount of CA corresponds to ~ / of a vesicle content per chromaffin cell The extreme consequence of the quantal theory should then be that a splanchnic nerve impulse volley should cause at random the secretion of one single CA vesicle (out of 13 000) in every third chromaffin cell

Let us assume then that under regular in vivo conditions the impulse frequency in the splanchnic nerve is ~1/ and under stress conditions ~10/s In case no resynthesis and no recapture of the secreted CA occurred the total adrenal CA store should be depleted within 10 and 2 h respectively In most experimental studies on nervous release of CA stimulation frequencies of 20–30 Hz have been used The chromaffin cells should under these conditions be depleted of all their CA content within half an hour

duction and inhibition of CA release may therefore be considered as due to non-energy requiring physico-chemical processes simple cation exchange playing an essential role The absence of diffusion barriers in the granule membrane renders it difficult to translate our present in vitro findings to in vivo conditions However in a forthcoming paper we will present arguments in favour of a nerve induced cation exchange instrumental in the release of CA from the adrenal chromaffin cells

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Fig 3 Schematic drawing of the proposed interplay between release pool and storage pool in the chromaffin granule (denoted pool 1 and pool 2 respectively). Pool 1 is a cation exchanger pool in which COO^- groups unselectively accept biogenic amines and inorganic cations. Pool 2 is specific for L-A and L-NA and its storing function is dependent on the ability of these two catecholamines to form weak COO^-—— NH salt bridges. Sodium ions are not able to enter this pool (for further details see Uvnäs & Åborg 1979b). (1) The two pools are empty. The COO^- groups in pool 1 are occupied by H ions. The COO —— NH_3 salt bridges are closed. (2) The two pools are filled with CA. In pool 1 the COO groups are occupied by CA. In pool 2 deionized COO^- and NH groups are occupied by CA and ATP respectively. (3) Due to nerve impulse induced influx of sodium ions such ions reach the COO^- groups of the cation exchanger pool and the CA is replaced by Na ions and released. (4) Due to the high concentration of CA in the storage pool CA from this pool immediately moves towards the COO^--groups of pool 1. Na ions will not accumulate in this pool until the CA pressure from pool is sufficiently reduced. The disturbed electroneutrality of pool 2 is reestablished by an equivalent release of ATP and the COO^-—— NH_3 salt linkage is closed. (5) With depletion of pool 1 sodium ions will increasingly accumulate in this pool

Our argumentation for a cation exchange dependent CA release from a two- (or three-)pool system in the chromaffin cell follows below

Importance of sodi m ion for CA relea

The entrance of sod m ions into chromaffin cell essential for CA release Recent reports suggest an essential role for Na ions in the CA release process. According to Banks (1967) and Banks et al (1968) perfusion of bovine adrenals with sodium-free Tyrode solution abolished or severely reduced the secretory response to carbamylcholine and to 70 mM KCl. Procedures assumed to increase intracellular sodium such as perfusion with potassium-free or ouabain-containing Tyrode solution increased the basal CA secretion. Veratridine induced CA secretion from perfused guinea pig adrenals was blocked by tetrodotoxin (TTX) presumably due to blockade of sodium channels opened by veratridine (Ito Nakazako & Ohga 1978). Knight & Whitaker (1978) reported that substitution of choline for sodium in the perfusion medium or addition of TTX abolished the CA release in isolated bovine medullary cells exposed to veratridine

The functional importance of sodium ions in the CA release process indicated by the above cited observations has led most authors to suggest that the opening of sodium channels and (or) the entrance of sodium ions should facilitate the entrance of Ca^{2+} ions a step which is commonly accepted as essential for the initiation of the release process. Our observations on the cation induced CA release (by calcium free isotonic NaCl solutions) from isolated chromaffin granules (Uvnäs & Åborg 1980) might rather indicate that the entrance of Na and Ca^{++} ions are parallel phenomena, sodium ions being responsible for cation exchange Na ⇌ CA at ionic sites in granules which—possibly under the mediation of Ca^{2+} ions—have become attached to the chromaffin cell membrane

Ca quant l adrenal CA secretion be explained s du t a intragranul cation xcha ge bet een CA and Na ions The electrophysical characteristics of the chromaffin cell membrane and the nerve membrane have been supposed to be widely different. The potential difference over the medullary cell membrane should be too low to allow any propagated action potentials only sufficing f r local minimal depolarization (Kanno & Douglas 1967). However recent observations on cultured medullary cells from rat (Brandt et al 1978) and from gerbil and adult human adrenal medullary cell (Biales Dichter & Tischler 1978) convincingly demonstrated resting potentials exceeding 50 mV as well as overshooting action potentials similar to those generated by sympathetic neurones. The action potentials disappeared either upon removal of external sodium ions or addition of TTX. The conclusion was drawn that the action potentials in the chro-

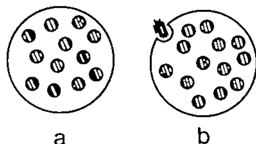


Fig 1 According to the current view CA secretion from the chromaffin cell is quantal the CA content of a vesicle being the smallest releasable quantum Together with CA all other soluble components of the vesicle are released (*a*) Resting chromaffin cell (*b*) Nerve impulse induced total discharge of the content of one CA vesicle.

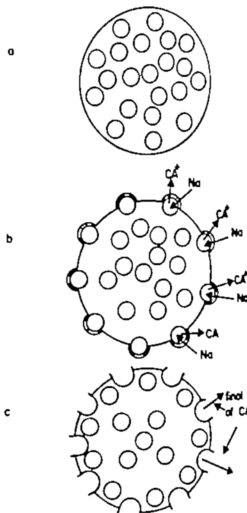


Fig 2 Proposed hypothesis of a quantal but multivesicular fractional release of CA from the adrenal chromaffin cell The size of the quantum of CA secreted depends on the nerve impulse induced sodium ion influx across the contact area between the chromaffin cell membrane and the vesicle ( ) Resting chromaffin cell with numerous CA vesicles adjacent to the plasma membrane (*b*) Nerve impulse induced depolarization of chromaffin cell membrane with attachment of vesicles to the membrane Sodium ion flux through open sodium channels result in CA release via cation exchange between CA and Na ions in granule cation exchanger (release) pool The fractional release allows a continuous refilling of the CA store by resynthesis ( ) Total evacuation of soluble vesicle components an infrequent phenomenon under regular in vivo conditions and occurring only as an ultimate step in order to dispose of worn out vesicles.

As well known the chromaffin cell has a considerable capacity to resynthetize CA However a secretory process that requires the rapid resynthesis of not only the hormone or transmitter in this case CA but also of all other storage components including matrix proteins and various synthetizing enzymes seems to be a rather wasteful machinery

We have in a series of papers reported on observations concerning a two-compartment storage of CA in the adrenal medullary granules (Uvnäs & Åborg 1977 1980*a* and *b*) The larger of these compartments capable of storing two thirds of the adrenal CA was specific—probably stereospecific—for A and NA The smaller of the compartments showed the properties of a weak cation exchanger unselectively accepting inorganic and organic (biogenic amine) cations Subsequent studies on a cation induced CA release from adrenal medullary granules in vitro suggested to us that this CA release was the result of a cation exchange (Na CA ions) in the cation exchanger pool—by us denoted the release pool—and that this release pool also served as a transfer pool for the depletion of the storage pool (see Fig 8 Uvnas & Åborg 1980*b*)

On the basis of the above-mentioned studies on the storage and release of CA and supported by recent observations in other laboratories on the significance of sodium ions for the release of adrenal CA we propose a hypothesis according to which the nervously (and possibly also the humorally) evoked secretion of adrenal CA is quantal although not with the total content of single vesicles as the smallest releasable quanta but with a concomitant fractional release of CA from multiple vesicles The size of the released quanta of CA is assumed to depend on a nerve impulse induced sodium ion flux and subsequent cation exchange (Na CA) across the contact area between the plasma membrane of the chromaffin cell and CA vesicles attached to this membrane The total evacuation of vesicles is considered as an ultimate under normal in vivo conditions rather infrequently occurring disposal of storage and building materials from worn out vesicles (Fig 2

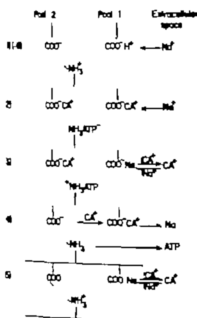


Fig. 3. Schematic drawing of the proposed interplay between release pool and storage pool in the chromaffin granule (denoted pool 1 and pool 2 respectively). Pool 1 is a cation exchanger pool in which COO groups unselectively accept biogenic amines and inorganic cations. Pool 2 is specific for L A and L NA and its storing function is dependent on the ability of these two catecholamines to form COO⁻—— NH_3 salt bridges. Sodium ions are not able to enter this pool (for further details see U nas & Åborg 1978b). (1) The two pools are empty. The COO⁻ groups in pool 1 are occupied by H ions. The COO —— NH_3 salt bridges are closed. (2) The two pools are filled with CA. In pool 1 the COO groups are occupied by CA and in pool 2 demasked COO⁻ and NH groups are occupied by CA and ATP⁻ respectively. (3) Due to nerve impulse induced influx of sodium ions such ions reach COO groups of the cation exchanger pool and the CA is replaced by Na ions and released. (4) Due to the high concentration of CA in the storage pool CA from this pool immediately moves towards the COO -groups of pool 1. Na⁺ ions will not accumulate in this pool until the A pressure from pool is sufficiently reduced. The masked electroneutrality of pool 2 is reestablished by dependent release of ATP and the COO⁻—— NH_3 salt linkage is closed. (5) With depletion of pool 1 sodium ions will increasingly accumulate in this pool.

Our argumentation for a cation exchange dependent CA release from a two- (or three-)pool system in the chromaffin cell follows below.

Importance of sodium ions for CA release

The entrance of sodium ions into chromaffin cells is essential for CA release. Recent reports suggest an essential role for Na ions in the CA release process. According to Banks (1967) and Banks et al (1968) perfusion of bovine adrenals with sodium-free Tyrode solution abolished or severely reduced the secretory response to carbamylcholine and to 70 mM KCl. Procedures assumed to increase intracellular sodium such as perfusion with potassium-free or ouabain-containing Tyrode solution increased the basal CA secretion. Veratridine induced CA secretion from perfused guinea pig adrenals was blocked by tetrodotoxin (TTX) presumably due to blockade of sodium channels opened by veratridine (Ito Nakazako & Ohga 1978). Knight & Whitaker (1978) reported that substitution of choline for sodium in the perfusion medium or addition of TTX abolished the CA release in isolated bovine medullary cells exposed to veratridine.

The functional importance of sodium ions in the CA release process indicated by the above cited observations has led most authors to suggest that the opening of sodium channels and (or) the entrance of sodium ions should facilitate the entrance of Ca^{2+} ions a step which is commonly accepted as essential for the initiation of the release process. Our observations on the cation induced CA release (by calcium free isotonic NaCl solutions) from isolated chromaffin granules (Uvnäs & Åborg 1980) might rather indicate that the entrance of Na and Ca^{2+} ions are parallel phenomena, sodium ions being responsible for cation exchange Na ⇌ CA at ionic sites in granules which—possibly under the mediation of Ca^{2+} ions—have become attached to the chromaffin cell membrane.

Can quantal and nonquantal CA secretion be explained as due to granule cation exchange between CA and Na ions. The electrophysical characteristics of the chromaffin cell membrane and the nerve membrane have been supposed to be widely different. The potential difference over the medullary cell membrane should be too low to allow any propagated action potentials, only sufficing for local minimal depolarization (Kann & Douglas 1967). However recent observations on cultured medullary cells from rat (Brandt et al 1978) and from gerbil and adult human adrenal medullary cells (Biales Dichter & Tischler 1978) convincingly demonstrated resting potentials exceeding 50 mV as well as overshooting action potentials similar to those generated by sympathetic neurones. The action potential disappeared either upon removal of external sodium ions or addition of TTX. The conclusion was drawn that the action potentials in the chro-

maffin cell membrane were due mainly to increased Na -ion permeability

During the millisecond of depolarization there will presumably be a free inward flux of Na ions across the chromaffin cell membrane Assuming that vesicles adjacent to the chromaffin cell membrane are attracted and attached to this membrane (due to the action of Ca^{2} ions entering concomitantly with Na ions?) the inwardly flowing Na ions will reach the membranes of the peripherally located granules and the postulated ion exchange Na ⇌ CA ions may occur Whether the prevailing permeability of the granule membrane for CA and Na ions suffice for the required ion passage or if the formation of semipermeable sites or opening of pores are needed can at present only be speculated upon However some reflexions as to the size of the ion flux that might be induced by a splanchnic nerve impulse across the contact surface between the chromaffin cell and the granule membranes might be possible from available data on sodium ion fluxes across other membranes

Hodgkin (1963) estimated the sodium ion flux in an unmyelinated giant nerve axon to about 20 000 and at a node of a myelinated nerve axon to about 300 000 ions/μm^2/impulse Caterall (1979) calculated the density of sodium channels in synaptosomes from rat brain to 25–50 channels/μm and the sodium ion flux/channel to 1×10^6 ions/min corresponding to a flux of 42 000–84 000 ions/μm /ms

With an estimated diameter of a chromaffin vesicle of $2\ 5\times10$ μm the surface of the vesicle (assumed to be spherical) will be

$$4\pi\left(\frac{2\ 5}{2}\right)^2 10\quad \mu m = 19\ 6\times10$$

(approx 2×10 μm) If we assume that the figures for the density of sodium channels and the size of the sodium flux in the chromaffin cell membrane approximate those of the synaptosomal membranes of the rat brain the sodium ion flux across an area of the chromaffin cell membrane of corresponding size should reach values around $2\times10\ \times42\ 500$–$85\ 000$ $=8\ 500$–$17\ 000$ ions/ms Calculated according to Hodgkin s figures for the unmyelinated giant axon (20 000/μm^2/ms) the corresponding ion flux across the chromaffin cell membrane should be $20\ 000\times 2\times10$ $=4\ 000$ ions/ms

Kirshner and Viveros have estimated the number of CA vesicles in a chromaffin cell to 13 000 and the CA content per vesicle to $7\ 2\times10^{-16}$ g corresponding to $2\ 4\times10^6$ molecules Since the CA release per nerve impulse was calculated to about / of the CA content of one vesicle (3) i e ~10^{3} molecules, a CA release due to cation exchange should require an equivalent amount of sodium ions to occupy the CA binding sites If during the exocytotic process, the contact area between a vesicle and the chromaffin cell corresponded to the whole vesicle surface and the sodium ion flux amounted to 10 000 ions/ms (see above) a minimum of 100 vesicles had to be engaged in the nerve impulse induced CA release 1 ‰ of the vesicle content being released by each impulse Probably the whole surface of the vesicle is not in intimate contact with the chromaffin cell membrane but on the other hand the number of vesicles concomitantly involved in the CA secretion might be considerably higher In fact with a mean chromaffin cell diameter of 1×10 cm and a mean vesicle diameter of $2\ 5\times10$ cm the inner surface of the chromaffin cell should more than suffice to accommodate all the 13 000 vesicles as a single submembrane layer With 10–20% of the vesicles concomitantly involved in the secretory process—and half of the vesicle surface in intimate contact with the chromaffin cell membrane the sodium influx and the consequent CA release should amount to less than one per mille of the CA content per secreting vesicle (1 per mille ~1 000 CA molecules)

The figures calculated for the nerve impulse induced sodium ion flux across the contact area between the chromaffin cell membrane and the vesicle membrane are rather speculative—to say the least—based as they are on several uncertain assumptions However they seem to us to indicate the possibility of a nerve impulse induced sodium ion influx during the millisecond of depolarization that might suffice for a multivesicular fractional release of CA based on a cation exchange as discussed in previous papers (Uvnäs & Åborg 1980)

Exocytosis with evacuation of the vesicles of the chromaffin cell a secondary instead of a primary event? A multivesicular fractional CA release on a cation exchange basis might seem difficult to reconcile with the many microscopical observations of exocytosis and expulsion of vesical contents into the exterior of the chromaffin cell One argument for an all or none response with a quantal release of whole vesicle packages might seem to be specially difficult to come around namely that CA and other vesicle components are reported to appear in perfusates or plasma in the same mutual proportions as in the chromaffin cell

However under the commonly prevail ng ex erimental conditions it has not been possible to study the chronological order in which the different granule components are released For the activation of the adrenal secretion humoral—usually acetyl- choline or carbamylcholine—or nervous stimuli (10 Hz or more) of unphy iologically high inten ties have usually been employed The conse uence of a high stimulation intensity w ll be a rapid depletion of the CA vesicles. Let us for the sake of discussion assume that ~3000 vesicles are con- tinuously engaged in the CA release On nervous stimulation ith a frequency of 20–30 Hz—a fre uency commonly used—the esicle stores will be depleted ithin 3 min Already with these moder te stimulation frequencies a possible dissociation of CA release and a discharge of the remaining esicle content will escape observation if—as usu lly done—sampling of perfusates and blood is made in 5- 10- 15-min periods or longer With the high doses of acetylcholine and carbamylcholine used as secretory stimuli the possibilities to observe chronological detail of the release proces have been even less. However in the few experiment where care has been taken to use stimuli of moder te intensity and (or) frequent sampling ha been made, a temporal dissociation has been observed between CA release and the appearance of protei (Banks et al 1967 Kirshner et al 1967 Dixon et al 1975) The retarded protein outflow has been interpreted as due to slow diffusion of proteins but the phenomenon might according to our in- terpretation as well be taken to indicate the sub- sequent emptying of CA depleted vesicles Recent ly Edwards et al (1979) reported that the output of dopamine β-hydroxylase was not proportional to the output of CA observed on splanchnic nerve stimulation in conscious cal es.

We therefore think that the available experimen- tal data might be in keeping with the idea of a cation (Na⁺) induced CA release as the primary event with the discharge of the remaining matrix of the vesicle as a second event It should be pointed out that with the low impulse frequencies prevailing under physiological conditions (~1/s) and the consider ble rate of resynthesis of CA assumed to exist (see Sjöstrand et al. 1977) complete emptying of a vesicle CA store and its ultimate consequence—the expul- sion of the vesicle matrix—might be a rather in- frequent phenomenon (Fig.)

As pointed out by Stjärne (1970) there are some observations which do not fit the idea of a quantal secretion based on the total evacuation of CA es- icles For example the quantal output of CA per nerve impulse from the cat adrenal gland depends both on stimulation frequency and on the total number of pulses i e the duration of stimulation On timulation of short duration there is an appar ently linear increase on prolonged stimulation a decrease of the CA secretion Such phenomena might be satisfactorily explained on the basis of a cation exchange dependent release of CA where the size of the released CA quanta will depend on the number of cations reaching the granule CA store and not on the total content of a esicle

Cation hange commun principle for the s r e and relea e of biogenic am ne We ha e pre iously shown that the release of histamine from mast cells can be explained as due to a cation ex change between extracellular sodium ions and in- tracellular histamine ionically bound to cationic sites (COO groups) in the granule matrix (for ref see U nas 1977)

In the present paper we ha e described a cation (N) dependent release of CA from adrenal medul- lary granules in itro and argued for a cation ex change dependent CA release being instrumental also on nerve impulse induced CA release n vo

We would in this connection like to call attention to the suggestion by U nas (1971) that also nervous transmitter release might be due to nerve impulse induced cation exchange According to Folkow Häggendal & Lisander (1967) e perimental data indicate that only about 3% (400 out of 15000 molecules) of the NA content of one adrenergic granule is released per stimulus from the individual nerve varicosity provided that each varicosity re leases transmitter every time an impulse arrives

If the calculations of Folkow et al. are correct it follows that the smallest releasable quantum of NA does not correspond to the content of a transmitter vesicle but only to a small fraction thereof Such fractional transmitter release from single or mult ple vesicles m ght be explained on the basis of a cation change due to nerv mpulse induced sodium flux across the nerve terminal–transmitter vesicle membranes at adrenergic as well as cholinergic nerve terminals. In fact prel minary n vitro obser vation suggest to us the occurrence of cation e change dependent storage and release of nervous transmitters in nerve granules These findings will be presented in forthcoming papers.

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Plasma concentration of neurotensin like immunoreactivity (NTLI) and lower esophageal sphincter (LES) pressure in man following infusion of (Gln[4])-neurotensin

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ROSELL, S, THOR, K, RÖKAEUS, Å, NYQUIST, O, LEWENHAUPT, A, KAGER, L & FOLKERS, K. Plasma concentration of neurotensin-like immunoreactivity (NTLI) and lower esophageal sphincter (LES) pressure in man following infusion of (Gln[4]) neurotensin. Acta Physiol Scand 1980, 109, 369–375. Received 15 Nov 1979. ISSN 0001-6772. Department of Pharmacology, Karolinska Institutet, Surgical Clinic, Ersta Hospital, Department of Medicine and Surgery, Huddinge Hospital, Stockholm, Sweden and Institute for Biomedical Research, University of Texas at Austin, Texas, USA.

(Gln[4])-neurotensin was infused for 5 to 70 min at 3 different infusion rates (6, 12 and 18 pmol kg × min respectively) in 19 male volunteers aged 26–47. The plasma concentration of neurotensin-like immunoreactivity (NTLI), the lower esophageal sphincter (LES) pressure, blood pressure, heart rate, ECG and blood glucose concentration were measured. The volunteers did not report any subjective effects during the infusion. Following infusion periods of 30 min or more the volunteers often reported bowel movements starting 5 min or more after cessation of the infusion. Neither blood pressure nor heart rate changed significantly. No changes were noted in the continuous ECG or in the blood glucose concentration. Apparent steady state levels of about 300 pM NTLI were reached at about 40 min during infusion of 12 pmol kg min^{-1} (Gln[4])-neurotensin. In all volunteers the LES pressure was significantly reduced within 5 min of starting the infusion. In 6 volunteers 1 pmol kg min (Gln[4]) neurotensin was infused for 5 min. The LES pressure decreased significantly ($P<0.01$) from 13.7±1.3 mmHg to 5.3±0.8 mmHg. The decrease in the LES pressure occurred at plasma NTLI concentrations of approximately 50 pM, at levels below those obtained in man after a meal or the ingestion of fat. The present data further support the hypothesis that in man plasma neurotensin, or a neurotensin metabolite is an endocrine hormone involved in the postprandial regulation of the motor functions of the gastrointestinal tract.

Key words: Neurotensin, lower esophageal sphincter pressure

In several species including man immunohistochemical investigations have shown that neurotensin, a tridecapeptide (Carraway & Leeman 1973, 1975), is stored in a specific type of cell (the N cell) which is present in the mucosa of the ileum and jejunum and to a lesser extent, in the colon and duodenum (Carraway & Leeman 1976, Orci et al. 1976, Sundler et al. 1977, Helmstaedter et al. 1977, Polak et al. 1977, Frigerio et al. 1977). Folkers et al (1976) suggested that (Gln)-neurotensin may be the naturally-occurring peptide rather than neurotensin. The biological tests which have been carried out so far show that the two peptides have the same biological activity both in vitro (Rokaeus et al. 1977) and in vivo (Andersson et al. 1976, 1977). In anesthetized dogs higher concentrations of neurotensin like immunoreactivity (NTLI) were found in blood draining from the ileum than in arterial blood (Mashford et al. 1978*a*), which indicates that neurotensin is released into the blood from the ileum. It has been shown in man that ingestion of a mixed meal caused a marked rise in the concentra-

tion of NTLI in blood (Besterman et al 1978 Mashford et al 1978*b*) and food appears to exert a major influence on the blood concentration of NTLI (Mashford et al 1978*b*) The ingestion of fat (Intralipid) is effective in increasing the plasma NTLI concentration whereas isocaloric amounts of amino acids (Vamin) and glucose have negligible effects (Rosell & Rokaeus 1979) Neurotensin administered by i v infusion in small doses to dogs inhibited gastric motility (Andersson et al 1977) and suppressed gastric acid secretion (Andersson et al 1976) It also increased blood flow in the gastrointestinal region and decreased it in subcutaneous adipose tissue (Rosell et al 1976) Taken together these data suggest that neurotensin or a metabolite thereof in the blood may be a hormone involved in the postprandial inhibition of gastric motility or gastric acid secretion and thus function as an enterogastrone (Mashford et al 1978*a* Rosell & Rökaeus 1979) However before neurotensin or a metabolite thereof can be ascribed such a physiological role it remains to be shown that NTLI concentrations in blood after the ingestion of fat are in the range found after the administration of doses of exogenous neurotensin capable of inhibiting motility in the gastrointestinal tract or gastric acid secretion in humans

The purpose of the present invstigation was to see if i v infusion of (Gln)-neurotensin has any effects on the lower esophageal sphincter (LES) pressure in man at NTLI plasma concentrations which have been reported after ingestion of fat The LES pressure was considered to be a suitable parameter to measure since ingestion of a fatty meal is known to cause a pronounced decrease in the LES pressure in man (Nebel & Castell 197) The effects of administration of neurotensin or (Gln)-neurotensin to man have not been reported earlier Therefore on the basis of data from toxicity tests in animals it was decided to monitor cardiovascular function by electrocardiography blood pressure measurements and heart rate determination Furthermore the plasma glucose concentration was measured

MATERIAL AND METHODS

The studies were performed in 19 male volunteers aged 26–47 with no symptoms of gastrointestinal disease or history of abdominal surgery The volunteers were asked to avoid eating fat in the evening before the experimental day and the experiment were started after a fasting period of at least 6 h between 9 a m. and p m. After a resting period of at least 30 min an indwelling cannula was inserted into the antecubital vein of one arm for blood sampling and another cannula was inserted into the other arm for the i v infusion of (Gln)-neurotensin

Systolic and diastolic blood pressures were measured by the cuff method at 5 min intervals from 30 min before to 40 min after the i.v infusion A continuous ECG approximately lead CR_4 was registered by an ink-jet recorder paper speed 10 mm×s throughout the investigation Blood samples were taken at 5 min intervals from 30 min before to 40 min after the i. infusion of (Gln)-neurotensin The samples were immediately placed on ice and later centrifuged at 4°C Two portions of each plasma sample were frozen and stored at −20°C until assayed (Gln⁸)-neuroten in was obtained from the same batch (No 14609 Institute for Biomedical Research University of Texas at Austin USA) as has been used for toxicity tests (T Malmfors unpublished). The substance was dissolved in sterile water under aseptic conditions and the solution was filtered (MFlex Millipore pore size 0 .. μm) directly into 5 ml vial (20 μg×ml) at 4°C (Kabi Pharmacy) The biological activity of a sample of this solution was compared with the activity of (Gln)-neurotensin in samples from the same batch which had not been filtered The biological activity was tested on the rat fundus strip (Rökaeus et al 1977) and wa found to be the same for the two samples Similarly the samples gave the same values in the radioimmunoa say for nuerotensin. Plasma glucose was measured in duplicate (Glox Kabi). Immunoreactive neurotensin (NTLI) was determined in duplicate in each plasma sample within 8 weeks after the experiments

The method used in the radioimmunoassay of neurotensin will be presented in detail elsewhere (to be published) and therefore only a brief description of the method is given here The antiserum (0-7701) was raised in rabbits by injection of neurotensin coupled to bovine serum albumin using 1-ethyl 3 (3-dimethyl-amino-propyl) carbodiimide HCl (Goodfriend Levine & Fasman 1964) The antigen was injected together with Freund complete adjuvant at 3–8 week intervals. This antiserum reacts with neurotens n (NT) NT (1 1) (Gln) NT and (Gln) NT (1 11) but not w th NT (4–13) or smaller C terminal fragments of NT It shows no cros -reactiv ty with the gastric inhibitory polypeptide (GIP), the vasoactive intestinal peptide (VIP) secretin cholecystokinin-33 cholecystokinin-39 pancreatic glucagon substance P somatostatin bovine pancreatic polypeptide bombesin gastrin-17 gastrin-34 or with trypsinized gastrin 34 which presumably contains the N-terminal tetradecapeptide Synthetic neurotensin (Beckman) labelled with I^{125} u ing the chloramine T method (Hunter & Greenwood 1962) The a say can measure levels down to 4 pM Experimental data indicate that some of the actions ascribed to neurotensin (vasoconstriction in adipose tissue) may be due to neurotensin metabolites (Rosell et al 1978) Since the radio-immunoassay is not specific enough to differentiate between neurotensin and its metabolite pharmacokinetic treatment of the plasma NTLI concentration data is limited to estimations of steady state concentrations and the terminal elimination

able 1 Plasma glucose concentration duri g infu ion of (Gln)- nrc t n in

...	Inf of (Gln)-NT			Plasma glucose M				
...	pmol	kg	min	Control	10 m	20 min	30 min	70 min
I	6			8.5	9._	8._	8 4	8 4
I	6			7 4	7 1	7 6	6 9	6 4
I	18			4 8	4 6	4 3	5.0	5 1
III	1			3 9	3.8	3 7	3.3	3 6
IV	1_			4 5	3 7	4 1	4.0	3 5
VI	6			4 4	4 8	4 9		
VII	6			9	8	_8		
VIII	6			3 4	3.3	3.5		
ion for 6 pmol kg min (n=5)				5.3	5 4	5 4		

...(t½) Calculation of t½ was made from the measured concentrations after deduction of the background concentration. Thus the t½ reflects the immunological clearance of the administered neurotensin. The half-life was calculated from the plasma NTLI concentrations ersus time curve by the method of least squares. The mean plasma NTLI level in 3 samples from the control period preceding the infusion period was taken as the background.

To measure the lower esophageal sphincter (LES) pressure esophageal manometry was performed in one series of expts ith 3 lumen manometric catheter assembly consisting of polyethylene catheters (inner diameter 1._ mm) ith 1.2 mm lateral openings 5 cm apart, connected external pressure transducers (Elema Schonander NT 3a). Pressure changes ere observed on an oscilloscope and recorded on multichannel photographic recorder (Elema Schönander Oscillograph 3006/DL). Each catheter was perfused ith distilled ater at constant rate of 1 2 ml min by Harvard constant infusion pump. With the subject in the supine position, the catheter assembly was passed by the transnasal route into the stomach and then slowly withdraw until the middle aperture as recording the LES pressure. Throughout each study the position of this aperture was maintained at point of maximum pressure. LES pressure was recorded millimeters of mercury using mean resting intragastric pressure as zero reference. The average pressure was determined from the recording during each minute of the test. In another series of expts   polyethylene catheter (inner diameter 3 5 mm) with 4 side holes (1.2 mm) at the same level was perfused at constant rate of 3 ml min⁻¹. It as passed by the transnasal route and withdrawn during suspended respiration at constant rate of 1 cm min from the fundus of the stomach to the esophagus. The intragastric pressure as used as zero reference. The tube as connected to the external transducer and pressure changes were observed on an oscilloscope and recorded on the multichannel photographic recorder. Each pull-through as carried out at 2.5 intervals.

Statistical evaluation of the data was performed with the Wilcoxon signed rank test and Student' *t*-test. The results are reported as mean ± standard error of the mean (M ± S E).

RESULTS

Cardiovascul act n plasma gluco and subject effect

(Gln)-neurotensin was administered i v for periods of 5 to 70 min at 3 different infusion rates 6, 1 and 18 pmol × kg × min   respectively. The systolic and diastolic blood pressures did not change significantly during or after the nfusion periods. Thus, the mean decrease in systolic and diastolic blood pressures 5 min after the start of the infusion of 12 pmol × kg × min was 5 9 and _ mmHg respectively (mean =11). The mean change in heart rate was 0 7 beats × min   Arrhythmias were not observed and no ST or T changes were noted in the recorded lead CR_4. The plasma glucose concentration did not change significantly (Table 1). The volunteers did not report any subjective effects during the infusion period. However following infusion periods of 30 min or more the olunteers often reported bowel movements starting 5 min or longer after cessation of the infusion and two of the volunteers had an urgent eed to de ecate within 30 min

Plasma level of NTLI

The plasma NTLI concentration curves following infusion of 12 pmol × kg × min (Gln)-neurotensin in two subjects are shown in Fig. 1 The initial part of the curves resemble zero order infusion curves. Apparent steady state levels of about 300 pM were reached at about 40 min The elimination slopes following the cessation of the infusions appeared to be multiexponential This was the case following infusion periods of 70 min ($n=3$) However following shorter infusion periods the elimina-

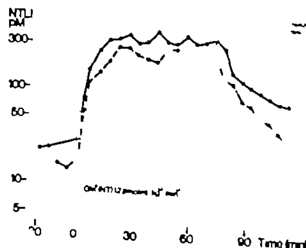


Fig 1 Plasma concentration of neurotensin-like immunoreactivity (NTLI) in two subjects in response to intravenous infusion of (Gln)-neurotensin 1 pmol × kg × min Vertical lines indicate the infusion period

tion slopes seemed to be monoexponential In experiments with infusion periods 5–30 min the $t\frac{1}{2}$ calculated on the first 4 to 6 post infusion determinations of plasma NTLI concentrations was 7 3±0 6 min (n=7) In 5 expts the infusion period was 70 min In those expts $t\frac{1}{2}$ was calculated on the first 4 to 8 NTLI determinations to be 9 4±0 9 min (n=5) the $t\frac{1}{2}$ calculated on the last 3–6 determinations was 19 9±1 min (n=3)

Lower esophageal sphincter pressure

13 pressure measurements in the lower esophageal sphincter were carried out in 1? volunteers receiving 6–18 pmol × kg × min (Gln)-neurotensin i v during 5–70 min In all volunteers the LES pressure was reduced significantly within the first 5 min of the infusion period Thereafter despite continued infusion of (Gln)-neurotensin there was a gradual but only partial return of the LES pressure to preinfusion values (Fig ?)

In 6 volunteers 12 pmol × kg × min (Gln)-neurotensin was infused i v for 5 min The LES pressure decreased significantly (P<0 01) from 13 7±1 3 mmHg to 5 3±0 8 mmHg with a rapid return to preinfusion values during the post infusion period (Fig 3) Thus in comparison with the preinfusion values there was no statistically significant reduction of the LES pressure 4 min after the cessation of the infusion The decrease in LES pressure occurred at plasma NTLI concentrations of approximately 50 pM (Fig 3) Apart from reducing the LES pressure (Gln)-neurotensin infusion also stabilized the nonrespiratory movements of the

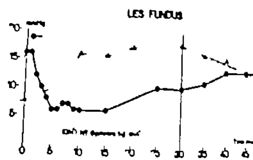


Fig Plasma concentration of NTLI (Δ) LES-pressure (●—●) in one subject in response to intravenous infusion of (Gln)-neurotensin 6 pmol × kg min for 30 min Vertical line indicate the infusion period

resting LES pressure seen in some volunteers 4) In 4 volunteers 1 pmol×kg ×min⁻¹ (Gln)-neurotensin was infused i v for 10 min. The pressure measured with the continuous perfusion through technique was reduced from 31 5± mmHg to 19±4 mmHg (n=4) (Fig 5)

DISCUSSION

The present series of experiments on healthy volunteers show that i v administration of (Gln)-neurotensin at low rates (6–18 pmol×kg⁻¹×min⁻¹) produces pronounced effect in the gastrointestinal tract The LES pressure decreased profoundly and the subjects frequently reported bowel movements

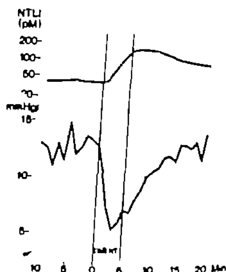


Fig 3 Plasma concentration of NTLI (upper curve) and lower esophageal sphincter (LES) pressure in response to intravenous infusion of 1 pmol × kg × min for 5 min The shaded area indicates the standard error of the mean (n=6) Vertical lines indicate infusion period

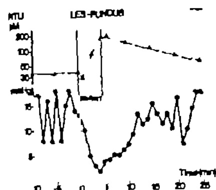


Fig. 4. Plasma concentration of NTLI and the LES pressure in one subject. The vertical lines indicate intravenous infusion of (Gln⁴)-neurotensin 12 pmol kg min

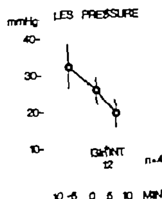


Fig. 5. The LES pressure as recorded with the continuous pull-through technique. Vertical lines indicate intravenous infusion of (Gln⁴)-neurotensin 12 pmol kg min. Means ± S.E. are indicated (n=4).

after cessation of the infusion and even an urgent need to defaecate. No significant changes in blood pressure, heart rate or in the continuously registered ECG were noted in this dose range. Moreover the plasma glucose concentration did not change. The actions in humans resemble the actions reported in dogs following i.v. infusions of neurotensin or (Gln⁴)-neurotensin in the same dose range (Andersson et al. 1976, 1977). Thus, studies carried out so far show that in both man and dog the most sensitive target organ for neurotensin is the gastrointestinal tract (Mashford et al. 1978b). Infusion of (Gln⁴)-neurotensin increased the plasma NTLI concentrations in an approximately dose-dependent manner. Infusion of 12 pmol × kg × min resulted in a plasma NTLI concentration of about 300 pM and after 18 pmol kg × min in one subject a level of about 500 pM was reached. These concentrations of NTLI are in the same order of magnitude as has been measured in man after a meal. Using the same antiserum as has been used in the assays in the present series, Mashford et al. (1978b) found that ingestion of meal after fasting for 24 h resulted in a mean plasma NTLI concentration of ~33 pM with a maximal value of about 600 pM in one subject. Consequently the effects on the LES pressure observed in the present investigation following infusions of 6–18 pmol kg × min occur at plasma NTLI concentrations which are found in man under physiological conditions.

Evidently (Gln⁴)-neurotensin does not have pronounced cardiovascular actions in man. On the basis of experiments on rat Carraway & Leeman (1973) suggested that neurotensin is a potent hypotensive and hyperglycemic peptide. However, to induce hypotension in dogs neurotensin has to be infused i.v. at about 10 times the dose necessary to induce inhibition of the motor activity in the gastro-intestinal tract (Rosell et al. 1976, Andersson et al. 1977). Similarly, to increase the plasma glucose concentrations in dogs neurotensin and (Gln⁴)-neurotensin must be administered in approximately the same doses that cause hypotension (Rosell et al. 1976). In the present expts. blood pressure, heart rate and plasma glucose level did not change. These quantitative comparisons indicate that in man, as in the dog, there is a clear-cut difference between the doses that produce gastrointestinal effect on the one hand and general cardiovascular effects and increased plasma glucose concentrations on the other.

The elimination of NTLI might possibly be multiphasic. However, a pharmacokinetic analysis of the plasma NTLI data should be postponed until it is known whether or not the radioimmunoassay is specific for administered peptide or also measures some of its metabolite(s) which may have different elimination rates.

The lower esophageal sphincter pressure was measured with the perfused catheter assembly positioned with lateral openings in the esophagus, the LES and the fundus of the stomach, respectively, or with pull-through technique with one perfused catheter. Both techniques have their advantages and disadvantages. The pull-through technique will measure LES pressure only momen-

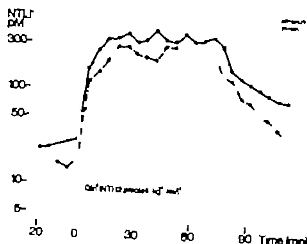


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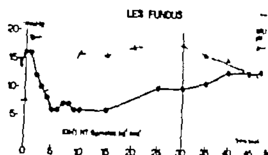


Fig 2 Plasma concentration of NTLI (Δ Δ) LES-pressure (O—O) in one subject in response to intravenous infusion of (Gln)-neurotensin 6 pmol kg min for 30 min Vertical lines indicate the infusion period

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Lower esophageal sphincter pressure

13 pressure measurements in the lower esophageal sphincter were carried out in 12 volunteers receiving 6–18 pmol × kg × min (Gln)-neurotensin i v during 5–70 min In all volunteers the LES pressure was reduced significantly within the first 5 min of the infusion period Thereafter despite continued infusion of (Gln)-neurotensin there was a gradual but only partial return of the LES pressure to preinfusion values (Fig 2)

In 6 volunteers 12 pmol × kg × min (Gln)-neurotensin was infused i v for 5 min The LES pressure decreased significantly ($P<0\ 01$) from 13 7±1 3 mmHg to 5 3±0 8 mmHg with a rapid return to preinfusion values during the post infusion period (Fig 3) Thus in comparison with the preinfusion values there was no statistically significant reduction of the LES pressure 4 min after the cessation of the infusion The decrease in LES pressure occurred at plasma NTLI concentrations of approximately 50 pM (Fig 3) Apart from reducing the LES pressure (Gln⁴)-neurotensin infusion also stabilized the nonrespiratory movements of the resting LES pressure seen in some volunteers (Fig 4) In 4 volunteers 12 pmol×kg ×min (Gln)-neurotensin was infused i v for 10 min. The LES pressure measured with the continuous pull-through technique was reduced from 31 5±6 mmHg to 19±4 mmHg ($n=4$) (Fig 5)

DISCUSSION

The present series of experiments on healthy volunteers show that i v administration of (Gln)-neurotensin at low rates (6–18 pmol×kg ×min) produces pronounced effects in the gastrointestinal tract The LES pressure decreased profoundly and the subjects frequently reported bowel movements

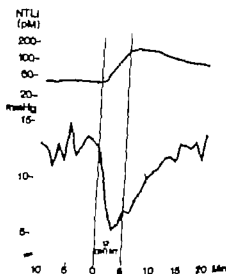


Fig 3 Plasma concentration of NTLI (upper curve) and lower esophageal sphincter (LES) pressure in response to intravenous infusion of 1 pmol × kg × min for 5 min The shaded area indicates the standard error of the mean ($n=6$) Vertical lines indicate the infusion period

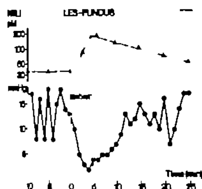


Fig. 4. Plasma concentration of NTLI and the LES pressure in one subject. Thin vertical lines indicate intravenous infusion of (Gln⁴)-neurotensin, 12 pmol kg

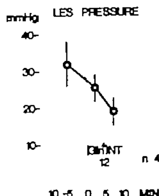


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tarily The resting LES pressure fluctuates (Bloom et al 1974) and is therefore difficult to determine with the pull through technique On the other hand with continuous registration of LES pressure the pressures are lower due to the low perfusion rate and the technique is not suitable for dose response studies but merely to find out if an effect can be registered or not However in the present study a decrease of the LES pressure after infusion of (Gln)-neurotensin could be demonstrated with both techniques

Nebel & Castell (1972) showed that ingestion of a fatty meal caused a pronounced decrease in the LES pressure in man This is interesting in view of our finding that fat is an important stimulus for the release of NTLI (Rosell & Rökaeus 1979) Therefore neurotensin or a neurotensin metabolite might be responsible for the decrease in the LES pressure after a fatty meal Several hormones released after food intake such as gastrin secretin cholecystokinin and glucagon have been shown to influence the LES pressure (Nebel & Castell 1972 Giles et al 1969 Castell & Harris 1970 Cohen & Lipshutz 1971 Resin et al 1973 Jaffer et al 1974) but it has not been established whether these hormones are of any physiological importance in the regulation of the LES pressure (Grossman 1973 Cohen & Lipshutz 1974 Sturdevant 1974 Henderson et al 1978)

We thank Professor John Wahren and his staff for placing laboratory facilities at our disposal

The study has been supported by the Swedish Medical Research Council (grant No 3518) AB Astra Research Foundation Stiftelsen Ruth och Richard Julins fund and the Robert A Welch Foundation and approved by the Ethical Committee at Karolinska Institutet

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Firing behaviour in nerve cell models with a two-state pore system

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SKAUGEN E. Firing behaviour in nerve cell models with two-state pore system Acta Physiol Scand 1980 109 377 392 Received 16 Nov 1979 ISSN 1000-677? Institutes of Physiology and Informatics University of Oslo Norway

The firing behaviour of simple nerve cell models with two-state pore system was investigated by computer simulation. The pores were assumed to open and close randomly with the probabilities for opening and closing calculated from the equations found by Hodgkin and Huxley for the squid giant axon. The cell models had cell body and an axon with an initial segment with smaller diameter Both the case of uniform membrane with constant pore densities all over the cell and the case of a cell body membrane with only leakage conductance (passive membrane) were investigated. The results indicate that the firing behaviour of small nerve cell may be significantly influenced by the finite number of pores in the initial segment. In contrast to the original Hodgkin–Huxley equations which give very non-linear frequency–current relationship in such nerve cell models, fairly linear relationship over large current range was found in many cases. It was estimated that the diameter of the initial segment must be less than approximately 1 μm and the length larger than half space constant, in order to obtain current frequency relationship significantly different from that predicted by the original Hodgkin–Huxley equations.

Key words. Hodgkin–Huxley membrane nerve membrane model stochastic pores

In two earlier papers it was shown that a finite number of pores or ionic channels in the nerve membrane could give stochastic fluctuations of the membrane conductances and the membrane potential (Skaugen & Walløe 1979 Skaugen 1980) Only the case of a space-clamped, uniform nerve membrane was considered. For sufficiently small pore numbers, less than approximately 20 000 pores, the firing behaviour of this space-clamped membrane was significantly influenced by these fluctuations. I shall here try to indicate how the stochastic fluctuations due to a finite number of pores may influence the firing behaviour of physiologically operating nerve cell. This was studied by simulating on computer simple nerve cell models with certain density of pores. The total number of pores in a nerve cell is much larger than the number 20 000 indicated above but what is important here is the number of pores around the site for spike generation. Usually this site is connected to the axon, and has a small diameter compared to the rest of the cell This will partly insulate it from the rest of the cell that is the axial resistance between the site for spike generation and the cell body or the dendrites and between this site and the axon is not insignificant compared to the membrane resistance of this site in the resting state The pore density in the initial segment is not known and its value can only be guessed at In different nerve fibres pore densities from 2.5 pores/μm^2 to several hundred pores/μm^2 have been measured (Colquhoun et al 1972, Keynes & Rojas 1974)

I have chosen to simulate simple model of nerve cells in order to study the "pure" effects of a finite number of pores without getting these effects mixed up with for instance the effects of a complicated dendritic network (with time delays reflecting of action potentials etc (Fernald 1971))

A realistic model of a nerve cell demands a detailed knowledge of the membrane properties of the whole neuron The membrane properties at the site for spike generation and at areas adjacent to it

...alent ...ial R, ...m)	Length of initial segment divided by space constant l/λ	Used in figures
	0 18	..3 5 6, 1
	0 41	2 to 10 and 1
)	0 91	to 6, 9 11 1
	0 18	..3 5 6 12
	0 41	to 10 and 1
)	0 91	2 to 6, 9 11 1
)	0 57	4
	0 41	8 9
)	0 91	9
	0 79	10
	0 18	10
)	0 64	11

a function of the injected current (in the cell body), for a few combinations of the size and the area of the cell, and of the pore densities in the initial segment. I either assumed that the density of pores in the cell body was equal to that in the initial segment, or that the nerve membrane of the cell body was purely passive. In the latter case a specific leakage conductance double of that in the initial segment was assumed in order to compensate for the lack of the potassium current, while the equilibrium potential for the leakage current was assumed equal to the resting potential. It seems that the dendrites of nerve cells do not in general conduct nervous impulses, which may mean that the nerve membrane behaves more and more like a passive membrane as one goes from the initial segment towards the dendritic branches (which can be explained by decrease in the pore densities).

The two cases considered here are thus extreme cases and the behaviour of a real nerve cell would be expected to lie between the behaviour shown by these two examples.

THEORY

The basis for these calculations is the now widely accepted assumption that the nerve membrane conducts sodium and potassium ions through pores, or ionic channels, and that the opening and closing of these pores are governed by stochastic process. As in the earlier papers no other types of electrical noise than conductance fluctuations are considered in the calculations (Skaugen & Walløe 1979, Skaugen 1980). The detail of the assumptions used here for the pore mechanisms were given in the first of these papers. For the readers con enience these assumptions are restated here.

1 The nerve membrane conducts ions through pores. In addition there is leakage conductance which is constant

2 A particular pore conducts only certain type of ion, either sodium or potassium ions.

3 All pores which conduct the same type of ion have the same conductance when in the open state.

4 There is no coupling between neighbouring gates or pores

5 Each pore has four gates. The pore is open only if all four gates are open

6. The transition probabilities of gate from one state to another either from shut to open, or from open to shut, are only potential dependent and given by the α and β parameters of the H–H equations.

7 The transition times of the gates are so short that they can be neglected. (The transition time is the time used by the gate to go from the shut to the open position or vice versa.)

The rest of the assumptions and the equations necessary to completely specify the space-clamped version of the model are given by Hodgkin and Huxley paper (Hodgkin & Huxley 1952)

In the modelling of whole nerv cell the usual method of dividing the cell into number of J compartments is used. I assume here that the compartments are cylindrical the j-th compartment having diameter d_j and length l_j Most often five compartments were used (J=5) Fig. 1 shows an approximation to typical vertebrate nerve cell with cell body tapering off to an axon with an initial segment which has smaller diameter than the rest of the axon.

One of the compartments, usually the middle one represented the main part of the initial segment. This compartment is termed the stochastic compartment, or the s-th compartment. In order to simplify the model sufficiently only this compartment was assumed to have finite number of pores. The initial segment has the smallest diameter and the smallest number of pores of the compartments. The inclusion of the effects of finite pore numbers for all compartments would increase enormously the number of pores taken into account, and it would make the computer time needed prohibitiv because the

Table 1 *Different nerve cell models used in the calculations*

Model	Dimensions of compartments when $R_i = 110\ \Omega$-cm diameter/length (μm) Compartment no 1	2	3	4	5	$\eta = \eta/\hat{\eta}$	Value of ϕ for $N_{Na} = N_K$ = 1 000	100	Pore density for N_{Na}, N_K 1 000 (pores/μm²)	100
A	16	0 43	0 2_	0 33	0 43	1 0	1 0	0 1	500	50
	2 92	0.59	9	? 9?	5 85					
B	1 ?7	0 25	0 13	0 19	0 25	1 0	1 0	0 1	500	50
	5	1	5	5	10					
C	0 74	0 15	0 07	0 11	0 15	1 0	1 0	0 1	500	50
	8.5	1 7	8 5	8 5	17 0					
A	6 84	1 37	0 68	1 03	1 37	1 0	0 01_	0 003	15 9	1 59
	?9 2	5 85	?9	?9	58 5					
B	4 00	0 80	0 40	0 60	0 80	1 0	0.03	0 003_	15 9	1 59
	50	10	50	50	100					
C	_34	0 47	0 _3	0 35	0 47	1 0	0 03	0 003	15 9	1 59
	85	17	85	85	170					
D	3 17	0 63	0 3	0 48	0 63	1 0	0 03	0 0032	15 9	1 59
	63	13	63	63	1?6					
E	1 4	0 80	0 40	0 60	0 80	1 0	0 012	0 003	15 9	1 59
	50	10	50	50	100					
F	7 _5	0 47	0._3	0 35	0 47	1 0	0 03	0 003	15 9	1 59
	85	17	85	85	170					
G	4 00	0 80	0 40	0 60	0 80	0 5	0 064	0.0064	15 9	1 59
	50	10	50	50	100					
H	4 00	0 80	0 40	0 60	0 80	0 2	0 16	0 016	15 9	1 59
	50	10	50	50	100					
I	34	0 47	0 _3	0 35	0 47	0 5	0 064	0 0064	15 9	1 59
	85	17	85	85	170					

All models which can be transformed into each other by the equivalent transformations given by Eq (4) are given the identification letter The pore density given in the table is the density in the initial segment (compartment no 1) The le L of this compartment in units of the space constant λ is also shown $\lambda \sim (d/(8 g_l R_i))^{\frac{1}{2}}$

are obviously of special interest Unfortunately the site for spike generation is generally of small dimensions compared to the rest of the cell and it is virtually impossible to measure the membrane properties here with the present-day techniques

For a few large nerve cells the membrane properties of either the axon or the cell body have been investigated by the voltage clamp method Well-known examples are the squid giant axon (Hodgkin & Huxley 1952) the myelinated nerve fibre of toad (Frankenhaeuser & Huxley 1964) and the myelinated nerve fibre of frog (Dodge 1963)

I have here chosen the Hodgkin–Huxley equations for the giant axon in squid as a basis for the calculations as in the preceding papers (Skaugen & Walloe 1979 Skaugen 1980) This choice is rather arbitrary there are a few other sufficiently detailed sets of equations for nerve membranes to choose from (Frankenhaeuser & Huxley 1964 Dodge 1963 Connor & Stevens 1971) The choice done here may be defended on the grounds that I am most interested in the region of a nerve cell where the action potential is generated These regions are unmyelinated and only the H H equations describe an unmyelinated nerve fibre Moreover the H-H equations are the simplest set of equations of the different sets we can choose from In practice this means a considerably shorter computational time than could be obtained by using any of the other sets of equations

Even using the H–H equations the present models were so complicated and time-consuming, mainly because of the introduction of a finite pore number that only a limited number of possible combinations of parameters could be explored A simple nerve cell with a cell body tapering off to an axon with an initial segment of smaller diameter than the rest of the axon was taken as the basic form of the nerve model The data are in most cases presented as the mean firing frequency (in the axon)

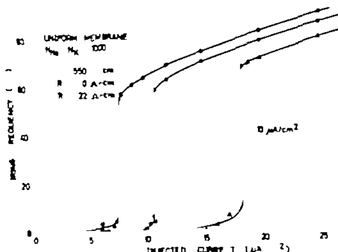


Fig. 3. Mean firing frequency as function of injected current for different values of R_i for model B in the case of uniform membrane and $N_{Na} = N_K = 1000$. The curve for R_i 22 Ω-cm drawn for I 10 µA/cm² less than true value.

where the symbols S and N indicate that E_s depends upon the total number N of pores in the s-th compartment and upon stochastic process S. The only difference from the previous models is that here the absolute values of the capacitance, the current, and the conductance are used, and that the "injected" current depends upon the potential in neighbouring compartments.

The values of all parameters used in these equations are kept constant and equal to their values in the H–H equations, except the rate constants and the maximal, specific membrane conductances. The values of all the rate constants are multiplied by φ and the values of the maximal conductances are multiplied by η. This transformation has been suggested by Huxley (1959) and it was used in a former paper (Skaugen 1980).

In the model the compartments are connected through a parameter I_j (or I_s) in Eq. (1). It is set equal to the current entering the j-th compartment. This current is the sum of the currents from the neighbouring compartments and the actual injected current. The latter is set equal to zero, except for the first compartment, the cell body. When I later speak of the injected current we mean the specific current injected in the cell body, that is, the total current injected here divided by the membrane area of the first compartment. The current I_j in Eq. (1) is thus given by

$$I_j = (E_{j-1} - E_j)g_j - (E_{j+1} - E_j)g_{j+1} + k_j I A_1 \qquad (2)$$

where

[illegible]

$E_0 = E_{n+1} = E_R$
$I_{n+1} = I, \; d_{n+1} = d$

where A_1 is the membrane area of the first compartment, and R_i is the specific internal resistance. The parameter g_j is the conductance between compartments j and j−1. The first compartment, the cell body, can be connected to the resting potential E_R by a conductance g_0 in order to simulate the loading of the cell body by an extensive system of dendrites. But in the cases presented here g_0 was set equal to zero and the soma and the dendrites were lumped together as one long compartment. In a similar way the last compartment, which is part of the axon, is loaded by a conductance g_L in order to simulate the continuation of the axon.

This model was tested against models where a short dendritic tree and a longer axon were included, but the results were not significantly different. The use of only one stochastic compartment probably introduced a larger error. I did not, however, test the model against a model with a very extensive dendritic tree where appreciable time delay effect might be expected as may for instance be the case in many motor neurons. I should perhaps emphasize here that we are only interested in the firing behaviour of the nerve cell, mostly the firing frequency as a function of the injected current and not, for instance, the conduction velocity of the action potential down the axon.

For a given geometrical configuration, with the diameters d_j and the lengths l_j of the cylindrical compartments specified, the pore numbers N_{Na} and N_K are given if the pore densities ρ_{Na} and ρ_K are known, $N_{Na} = \pi d_s l_s \rho_{Na}$ and $N_K = \pi d_s l_s \rho_K$ where s indicates the stochastic compartment. In the model we need not specify the pore densities, but the pore numbers must be known. With these numbers, the geometry of the cell and all parameters in the H–H equations given, the model is completely specified.

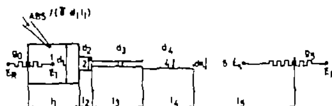


Fig 1 Diagram of nerve cell model with 5 compartment of length l_j and diameter d_j Membrane potential E_j calculated at the mid-point of each compartment Internal conductances g_j are taken between the mid-points of the j-th and the (j+1)-th compartment Current is injected in the soma The value I used in the figures is the absolute value I_{abs} divided by the membrane area $\pi d_1 l_1$ of the soma E_R is the resting potential

speed of computation for each compartment is proportional to the number of pores between approximately 1 000 and 20 000 pores constant for larger pore numbers (Skaugen & Walløe 1979)

It is however felt that the simplification used here is an acceptable approximation The compartments on the "input" side of the initial segment including the cell body have such large pore numbers that the fluctuations of the membrane potential will here be much smaller than in the initial segment and therefore of much less importance The compartments on the "output" side of the initial segment represent the axon and have little effect upon the generation of the action potential in any case It is expected that the effects of finite pore numbers in the model would have been somewhat enhanced if all compartments had been described by the stochastic model The results presented here can therefore be considered as lower limits for these effects.

Another source of error is the division of the nerve cell into rather few compartments (usually 5) This was tested by a model where all compartments were governed by the H H equations (no stochastic compartment)

The initiation of an action potential in the cell body and its time course in all the five compartments were calculated for different time step lengths Δt in the numerical solution and for the three geometrical configurations used in most of the stochastic calculation (see Table 1) As an example of this Fig shows the membrane potential as a function of time in the third compartment for step lengths $\Delta t = 0.1$ ms and 0.05 ms (continuous lines) This can be compared against the broken line which shows the potential for the same configuration but with fifteen segments and $\Delta t = 0.005$ ms The injected current (in the cell body) is in all cases shown here 10 μA/cm Each of the 5 segments in the first case is now divided in 3 segment Any further reduction of Δt did not change the result more than the line width in the figure In all the three cases shown the curve for 5 segments and $\Delta t = 0.05$ ms is very close to the broken curve The error introduced in the time (the time delay of the action potential) is always less than 0.1 ms for the value of the injected current used here. The time delay is larger near the current threshold for firing, but in all cases it corresponds to a variation in the injected current of less than 5% (A small

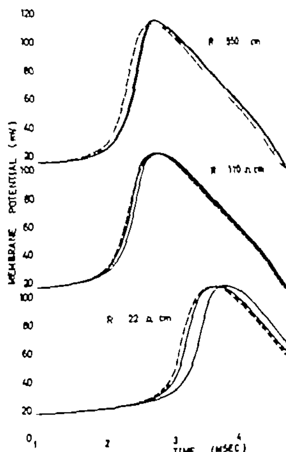


Fig 2 Membrane potential at the mid point of the initial segment as a function of time for a non-stochastic calculation of model B for different values of R_i I 10 µA cm² In each case the potential was calculated for 1 compartment and a step length Δt 0.005 ms (broken curve) and for 5 compartments (unbroken curves) with $\Delta t = 0.1$ ms and 0.05 ms The curve for Δt 0.05 ms is closest to the broken curve

decrease of the current of this amount would give the same time delay) It is felt that this is accurate enough for our purposes Five segments in the nerve cell model and a time step length $\Delta t = 0.05$ ms are thus sufficiently accurate in the non-stochastic numerical calculation The value of Δt was used as the maximum step length Δt_{max} in the stochastic calculation and it was tested and found sufficiently accurate here also (Skaugen & Walløe 1979)

In the stochastic compartment the membrane potential E_s is given by a set of equations similar to the ones describing the stochastic model presented before for a space-clamped nerve membrane In all the other compartments the membrane potential E_j is given by the H-H equations For all the compartments including the stochastic one these equations describe the membrane potential E as a function of time t and the injected current I

$$E_s = E_s(t, I, N, S) \qquad (1)$$
$$E_j = E_j(t, I) \quad j \neq s$$

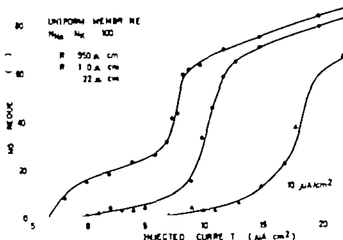


Fig. 5 Mean firing frequency as function of injected current for different values of R_i for model B in the case of uniform membrane and $N_{Na}=N=100$. The curve for R_i 22 Ω-cm draw for 10 μA/cm² less than true value.

...ked notation for changing the geometrical configuration of the nerve cell

The basis for the numerical solution of the equations in the stochastic compartment is the method termed method 2b in previous paper (Skaugen & Walløe 1979). This method as tested and found sufficiently accurate for maximum step length Δt_{max} of 0.05 ms, which is also used here. The step length Δt is generated by stochastic process this method (it is the time interval between two changes of the membrane conductances, limited upward to Δt_{max}). It can have any value between 0 and Δt_{max}. It the step length used for all the other compartments.

For the stochastic compartment the time interval Δt divided in two, in order to include the effects of the current I_s in Eq. (1). In the first half of the interval the potentials E_i and E_{i+1} in Eq. (3) at the beginning of the time interval are used. In the latter half the values at the end of the interval are used.

For the non-stochastic compartments the basis for the numerical solution is faster version of the method used by Hodgkin and Huxley in the first solution of their equations (Hodgkin & Huxley 1952). This was found to be the best of number of methods tested and in addition an extremely stable one. Values of E_i are first predicted by predictor formula. These values are then used in corrector formula to predict new and better values of E_i. This repeated once. Briefly stated, the whole numerical calculation is as follows.

We are at time t_0 with the alues of all parameters known. These are indexed by 0. We are going to find the values of all parameters at time $t = t_0 + \Delta t$. These are all indexed by 1.

1. By the drawing procedure described before (Skaugen & Walløe 1979), draw the time Δt to the next change of conductances in the stochastic compartment.

2. If $\Delta t > \Delta t_{max}$, set $\Delta t = \Delta t_{max}$ and skip numbers 11 and 12.

3. Predict alues of E_j at time $t_1 = t_0 + \Delta t$: $E_{j1} = E_{j0} + (dE_{j0}/dt)\Delta t$ for $j \neq s$.

4. Predict a value of E_{s1}: $E_{s1} = EE_s + (E_{s0} - EE_s)\exp(-(G/C_s)\Delta t)$, where $G = g_{Na} + g_{K} + g_{L} + g_{-} + g_{+}$, $EE_s = (g_{Na}E_{Na} + g_K E_K + g_L E_L + g_- E_{-} + g_+ E_{+})/G$. g_{Na} and g_K are the sodium and potassium conductances of the stochastic membrane and E_{Na}, E_K and E_l are the equilibrium potentials for sodium, potassium and for the leakage conductance G_L. C is the membrane capacitance.

5. Values of the α and β parameters in the H–H equations are calculated for all compartment using the values of E_{j1}.

6. Values of the h, m parameters in the H–H equations are calculated by the corrector formula.

$$h_{j1} = (2h_{j0} + (\frac{dh_{j0}}{dt} + \alpha_{h1})\Delta t)/(2 + (\alpha_{h1} + \beta_{h1})\Delta t),$$

and correspondingly for m_{j1} and n_{j1}.

7. The alue E_{s} at the middle of the interval Δt is calculated.

$$E_{s} = EE_s + (E_{s0} - EE_s)\exp(-\tfrac{1}{2}(G/C_s)\Delta t)$$

8. Calculate new values of E_{j1} ($j \neq s$) by the corrector formula

$$E_{j1} = (2C_jE_{j0} + (C_j\frac{dE_{j0}}{dt} + \bar{g}_{Na}h_{j1}m_{j1}^3 E_{Na} + \bar{g}_{K} \ldots E_K$$

$$g_L E_L + g_- E_- + g_+ E_+)\Delta t)/(2C_j + (\bar{g}_{Na}h_{j1}m_{j1}^3 + \bar{g}_K n_{j1}^4 + g_L + g_- + g_+)\Delta t).$$

9. Calculate new value of E_{s1}

$E_{s1} = EE_1 + (E_{s} - EE_1)\exp(-\tfrac{1}{2}(G/C_s)\Delta t)$, where $EE_1 = (g_{Na}E_{Na} + g_K E_K + g_L E_L + g_{-1}E_{-} + g_+ E_+)/G$

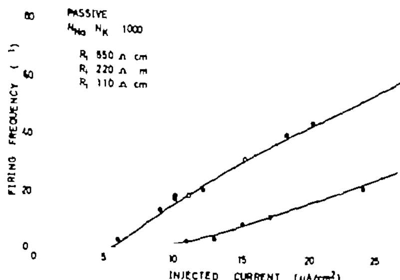


Fig 4 Mean firing frequency as function of injected current for different values of R_i for model B in the case of a passive soma and $N_{Na} = N_K = 1000$

The same model equations can however be obtained *from several different* sets of parameters for the nerve cell Assume that *all the rate constants are multiplied by* ϕ all the maximal membrane conductances are multiplied by η *the specific* internal resistance is multiplied by w *all the diameters* are multiplied by r all the segment lengths are multiplied by q and the conductances of *single pores are* multiplied by u The basic equations for the model can then be written as

$$C\frac{dE_j}{d\tau} = -\frac{\eta}{\phi}\Big(g_{Na}(E - E_{Na}) + g_K(E_j - E_K) + g_l(E - E_l)$$

$$+ k_j \frac{1}{\eta} + \frac{r}{\eta w q^2}\frac{I_{sj}}{A_j}\Big) \qquad (3)$$

where

$\tau = \phi t$ $A_j = \pi d\, l_j$

$g_{Nx} = g_{Nx}(\tau\ \alpha\ \beta_x\ S)$ x = h or m

$g_K = g_K(\ \alpha\ \beta\ S)$

$$N = \frac{\eta r q}{u} N_s$$

and where C g_{Na} g_K and g_l are the specific membrane capacitance and the specific sodium potassium and leakage membrane conductances respectively E_{Na} E_K and E_l are the equilibrium potential for sodium potassium and the leakage iones respectively The currents and I_{sj} are defined in Eq (2) g_{Na} and g_K are functions of the transformed time τ the rate constants α and β_x which are functions of the potential E only and of a stochastic process *S for j = s N* and N indicate the *number of pores in the stochastic compartment before and after the transformation.*

We see that the equations are left formally unchanged if $\phi = \eta$ $r = \eta w q^2$ and $u = \eta r q$ The injected current depends upon η but as it is a free variable it can be adjusted correspondingly Instead of the real time t the transformed time $\tau = \phi t$ is used which means that the frequencies calculated and presented here must be multiplied by ϕ

In a former paper it was shown that ϕ probably was roughly equal to η over a wide range of values of (Skaugen 1979) This will be assumed to be the case here The first condition $\phi = \eta$ is then satisfied and the conditions for formally unchanged equations can be written as

$$r^3 = \frac{w}{\eta}u^2 \quad q = \frac{u}{wq^2} \qquad (4)$$

$$\phi = \eta$$

If the conductances for single pores are assumed constant the parameter u is constant and r q and ϕ depend upon w and η only In a given nerve cell model the pore density ϱ and the specific internal resistance R_i can thus be changed to arbitrary new values $\eta\varrho$ and wR_i without changing the firing behaviour If the diameters and lengths of the model compartments are multiplied by the r and q given by Eq (4) In the same way the assumed conductances for single pores can be changed Simulation of a single specified model thus covers a wide range of possible models

Equation (4) also shows that a change in one parameter corresponds *to changes in other parameters* I found it convenient to investigate different cell models by changing the specific internal resistance R_i from 22 Ω-cm to 550 Ω-cm *If R = 110 Ω-cm* is taken as the standard size w is then changed from 0 *to 5* This corresponds to keeping R_i constant at its standard value while the compartment diameters and lengths are changed by factors ranging from $r = 1/\sqrt[3]{0.2} = 1.71$ and $q = \sqrt[3]{0.2} = 0.585$ to $r = 1/\sqrt[3]{5} = 0.585$ and $q = \sqrt[3]{5} = 1.71$ This latter change of the cell geometry is more interesting but also more complicated to keep track of We may thus take the simple change of the internal specific resistance as a short-

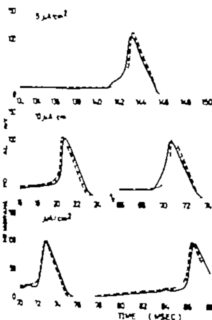


Fig. 7 Membrane potential as function of time in soma (broken curve) and in initial segment (unbroken curv) for different values of the injected current. Note break in middle curve (10 µA/cm²)

then be multiplied by ϕ and η as explained before The sodium and potassium pore densities were assumed equal, and the total numbers N_{Na} and N of pores ere given the values N_{Na}=N =100 and N_K N 1000

Figs 3 and 4 show the mean firing frequency f as a function of the injected current I when N_{Na}= N_K 1000. For each figure three different values of the specific internal resistance R_i are used Ac cording to the equivalent transformations described by Eq. (4) this corresponds to three different geometrical configurations with R_i=110 Ω-cm as shown by Table 1

The case of uniform membrane is shown by Fig 3 that is, the maximal specific membrane conductances are equal in all the different compartments representing the cell body the initial segment, and the axon The frequency-current relationship is ery close to the relationship obtained in a non-stochastic nerve cell model where the potential in all compartments is calculated by the original H-H equations In this case the mean firing frequency f increases ery rapidly from zero to approximately 55 s w en the injected current I is increased. Thi happens at the threshold current for firing. The slight increase of the firing frequency f above zero at somewhat smaller alues of the cur rent which is shown by Fig. 3 is thus absent in a non-stochastic model But in spite of this d fference the overall effects of a finite pore number upon the firing behaviour are very small in this case

This is quite different in the case of a cell body (or soma) with a pa sive membrane that is in the soma there is only a leakage conductance which is constant and independent of the membrane potential As shown by Fig. 4 there is now a much more linear frequency-current relationship This is not the case in the corresponding non-stochastic model where the frequency increases rapidly from zero to a high value when I is increased above a certain threshold alue This corresponds more to the curves shown by Fig. 3

The difference between the two cases shown by Figs 3 and 4 is not difficult to understand For uniform membrane the soma can fire but the initial segment has a lower threshold for firing than the soma due to the stochastic fluctuations of the membrane conductances (Skaugen & Walloe 1979) On the other hand the current is injected in the soma, and the mean membrane potential therefore is somewhat higher there than in the initial segment As the injected current I is increased the (stochastic) initial segment starts to fire but for slightly larger values of i the threshold current for the non-stochastic soma is reached and the soma starts to fire at a high frequency as in the original H-H equations (Hodgkin & Huxley 195) In the uniform membrane model the threshold current is 6.3 µA/cm² but here the soma is loaded by the initial segment and the axon and part of the injected current goes to these compartments. As the internal resistance is decreased the load is increased, and the threshold current for firing is increased as shown by Fig. 3

When the soma is passive however it cannot generate an action potential and the firing be ha iour is more determined by the properties of the stochastic initial segment For not too large pore numbers (≤20 000) the frequency-current relationship for the tochastic segment is distinctly more linear than for the original H-H equations, as shown before (Skaugen & Walloe 1979 Skaugen 1980) This give the fairly linear frequency-current relationship for the whole nerve cell. Since the membrane potential of the initial segment is only indirectly affected by the injected current (via the

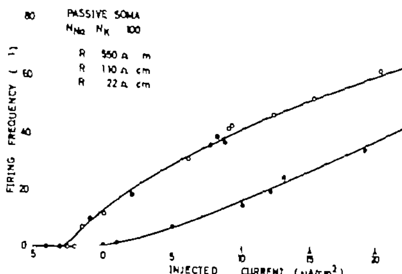


Fig 6 Mean firing frequency as function of injected current for different values of R_i for model B in the case of a passive soma and $N_{Na}=N_K=100$

10 Repeat step 5 6 7 8 and 9

11 By the drawing procedure described before (Skaugen & Walloe 1979) draw what the changes in the membrane conductances g_{Na} and g_K of the stochastic compartment are Change these conductances accordingly

12 We are now at time t with the values of all parameters known Go to step 1 and repeat the procedure

In addition to this the potential of the s-th compartment will be calculated by the H-H equations as in the other compartments during an action potential as described earlier (Skaugen & Walloe 1979).

In the actual program every care was taken to avoid unnecessary operations in the computation loop shown here The quantities used in the actual program were therefore combinations of the symbols shown here and the expressions were considerably simpler The greatest saving in computer time was obtained in step 5 Before the start of the simulation the values of the α and β parameters in the H H equations were calculated for E equal to E_K-1 mV in steps of 1 mV until $E_{Na}+1$ mV and then stored in an array In step 5 the values are found by direct linear interpolation between the values stored in this array

The results of the simulations depend upon a stochastic process (employing two quasi-random number generators) and for the same parameters different simulations (starting with different seeds in the random generator) will give values distributed around a mean value The standard deviations of the results were calculated but not shown in the figures to avoid cluttering. The scatter of the simulation results around the curves will however indicate the amount of uncertainty The curves are drawn by hand as the best fit to the simulation result

The models were usually simulated for one second of model time but some times for two seconds at low firing frequencies This time was too short to obtain reliable estimates of the standard deviation of the interspike intervals the burst factor etc as was done for the membrane models described earlier (Skaugen & Walloe 1979 Skaugen 1980)

It should be noted that in a non-stochastic nerve cell model based only upon the original H-H equations, the membrane potential will always be highest at the point of current injection when there is no firing. In the model shown in Fig. 1 a current will flow axially from the soma towards the axon as the latter is closer to the resting potential (A depolarizing injected current is assumed) As the current flows towards lower potentials, the potential must decrease from the soma toward the initial segment and the axon. In the case of a uniform membrane the threshold for firing will therefore be reached first in the soma if the injected current is increased. The spike will therefore always start in the soma. This is generally not the case in the stochastic models described here. In a non stochastic model the spike will start in the beginning of the axon only if the threshold for firing is somewhat lower than in the soma The axon membrane must then have some properties different from those of the soma membrane as for example a shift in the potential dependence for sodium conductance or a higher density of sodium conducting pores

RESULTS

Table 1 shows examples of the nerve cell models used here for a few different values of ϕ assuming u = 1 and a sodium pore density of 500 pores/μm² in squid All the actual simulations were performed with $\phi=1$ such that the value of η shown in the figures is the value of $\eta=\eta/\phi$ if we use the results for $\phi\neq1$ The frequencies and currents shown must

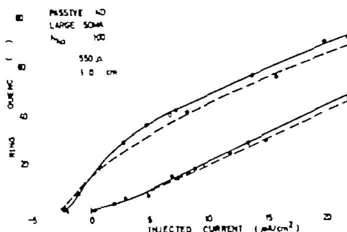


Fig 9 Mean firing frequency as function of injected current for different values of R, in the case of passive soma for large soma (unbroken curve model E) and for normal soma (broken curve model B) $N_{Na}=N_K=100$.

μA/cm² the spike is in most cases generated first in the initial segment and then spreads up to the soma and down the axon as shown for i=5 μA/cm² in the figure. It should be noted that in a nerve model based wholly upon the original H–H equations the spike will always start at the soma, at the point of current injection, in the case of a uniform membrane

On the steep middle part of the curve from approximately 8 to 13 μA/cm² the spike may arise first in either the soma or the initial segment as shown by the two neighbouring action potentials for i=10 μA/cm² On the upper part of the frequency current relationship the spike is in most cases generated first in the soma as shown for i=15 μA/cm² The soma is now fired directly by the injected current and the potential in the initial segment just follows

For a real nerve cell where the excitability of the membrane probably decreases from the initial segment toward the dendrites, the tendency for the spike to start at the initial segment will be stronger The cell then lies closer to the case of passive soma where the spike always is generated in the initial segment.

In the following figures some parameters are changed to obtain model which cannot be obtained by the equivalent transformations described here. Figs. 8 and 9 show the frequency–current relationship when only the soma is increased in size (continuous line) as compared to the former case (broken line) For the uniform case shown by Fig 8 the lower part of the two curves where the spike is generated in the initial segment are almost identical But the larger soma is relatively less loaded by the initial segment and the axon and it firing behaviour is less affected and closer to that of the isolated space-clamped membrane with its steep increase of the frequency around the current threshold for firing This difference is clearly seen in the upper part of the curve.

For the case of the passive soma shown by Fig. 9 the difference between the large soma (continuous line) and the former case (broken line) is very small The larger soma is able to maintain a slightly larger membrane potential at a certain value of the injected current because it is relatively less loaded This gives slightly larger value of the axial current reaching the initial segment where the firing is started But the resulting increase in the firing frequency is hardly significant For the passive soma the size of the soma thus seems to influence the firing behaviour very little when the cell body is not too small.

The effects of reducing the maximal membrane conductances without changing the rate constants are shown by Figs. 10 and 11 For a constant pore conductance (=1) this corresponds to a similar reduction of the pore densities The condition $\phi=\eta$ in Eq (4) now no longer holds true and the equiv

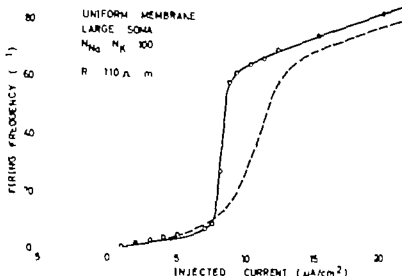


Fig 8 Mean firing frequency as function of injected current for R_i = 110 Ω-cm in the case of a uniform membrane for a large soma (unbroken curve model E) and for a normal soma (broken curve model B) $N_{Na} = N_K = 100$

soma) and also due to the load of the axon the effective current exciting the initial segment is considerably smaller than the injected current i shown in the figures The firing frequency therefore increases slower when i is increased than found for the uniform membrane The special geometry of the nerve cell thus helps to make the frequency-current relationship more linear over a larger current range This is clearly seen by comparing Fig 4 and the curve for N_{Na} or N_K = 1 000 in Fig 4 and 5 in an earlier paper (Skaugen & Walloe 1979) But this depends upon the stochastic properties of the initial segment In a non-stochastic nerve cell model the initial segment has a sharp threshold for firing and this will give the sudden increase in the firing frequency so characteristic for the H–H equations

As the specific internal resistance R_i is decreased the initial segment is more heavily loaded by the soma and the axon and at a certain point an action potential can no longer be generated because the current lost axially is too large This happens for R_i = 110 Ω-cm in Fig 4

Figs 5 and 6 show the firing frequency as a function of the injected current for the smaller pore numbers $N_{Na} = N_K = 100$ In the case of a uniform membrane the threshold for firing in the initial segment is now sufficiently small to affect the firing behaviour considerably The sharp threshold for firing which was seen for 1 000 pores is now seen as the steep middle part of the curve while the lower slowly rising part of the curve has become much larger This gives the frequency-current relationship a distinct sigmoid shape The lower part of the curve can be reasonably well approximated by two straight lines one with a small slope for small values of the injected current and one with a larger slope for larger values This resembles the primary and secondary firing range observed in some nerve cells (Kernell 1965) but other mechanisms have been found which can explain this (Kernell 1971)

For a passive membrane in the soma the firing behaviour for $N_{Na} = N_K = 100$ as shown in Fig 6 is much more similar to the behaviour shown in Fig 4 for larger pore numbers The frequency–current relationship is fairly linear and its slope is small The main difference between the cases shown by Figs 4 and 6 is a current threshold for firing which is approximately 10 $\mu A/cm^2$ smaller in Fig 6 than in Fig 4 There is also a firing response for smaller values of the specific internal resistance R_i for the smallest pore numbers

The action potential is always initiated in the initial segment when the soma membrane is passive because the soma then cannot fire The situation is more complex in the case of a uniform membrane Fig 7 shows typical examples of action potentials as registered in the initial segment (continuous line) and in the soma (broken line) in the standard model for R_i = 110 Ω-cm shown in Fig 5 On the slowly rising lower part of the curve to approximately

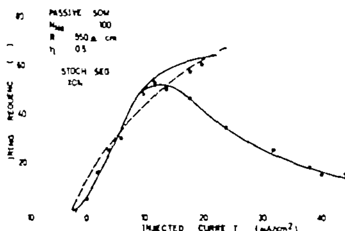


Fig. 11. Mean firing frequency function of injected current for $R_i = 550$ Ω-cm and for η 0.5 (unbroken curves) and $\eta = 1.0$ (broken curve). Open and filled circles represent firing in the initial segment and in the axon, respectively. $N_{Na} = N = 100$. Passive soma.

...form H-H equations the frequency–current relationship was quite linear over a considerable range of the injected current in the case of a passive soma. In fact, it was more linear than for the simpler membrane model discussed before (Skaugen & Walløe 1979, Skaugen 1980). It is thus seen that the geometry of the nerve cell can be important for its firing behaviour.

The nerve cell models were not simulated with pore numbers larger than $N_{Na} = N = 1000$ in the initial segment. But the results obtained for the isolated membrane showed that a significantly more linear frequency–current relationship than for the H-H equations was obtained for pore numbers up to 20000 (Skaugen & Walløe 1979). Since the geometry of the nerve cell increases this linearity in the case of a passive soma we may expect significant differences from a non-stochastic model at least up to this pore number. As previously noted the simulated model time was too short to obtain reliable estimates of the variability of the interspike intervals, but some general comment can be made. In the case of a uniform membrane the firing was clearly very regular for frequencies higher than 50–60 which corresponds to the threshold for firing in the Hodgkin–Huxley model. For lower frequencies and in cases of passive soma the standard deviation ΔT of the interspike intervals was not significantly different from the relation found in the membrane model described in an earlier paper (Skaugen & Walløe 1979, their Fig. 8) where $\Delta T = 0.8/f$ for low firing frequencies in models with pore numbers less than 10000. The discharge will in these cases be quite irregular. For instance, at a mean firing frequency f of 10 s the interspike intervals T would mainly lie in the range $T = 1/f - \Delta T = 20$ ms to $T = 1/f + \Delta T = 180$ ms.

It thus seems that for neurones firing quite regularly at a low frequency the stochastic processes described here would not be of any major importance for the observed frequency–current relationship. A low regular firing frequency is then probably due to a nerve membrane with somewhat different properties from those described by the Hodgkin–Huxley model as for instance in the membrane model suggested by Connor & Stevens (1971) where low firing frequencies could be generated.

The burst firing reported for the stochastic membrane model was not investigated here mainly because it was connected with higher pore numbers than used here but also because the model time used in the simulation (1 s) was too short to investigate finer details in the distribution of firings in time. From the results obtained however no tendency to burst firing could be seen for the case of passive soma. For the case of a uniform membrane there was a tendency to burst firing for small frequencies (at the foot of the curves in Fig. 3) but only for the largest pore numbers used

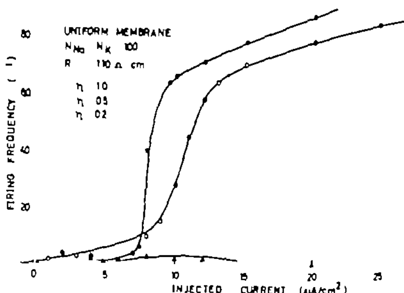


Fig 10 Mean firing frequency as function of injected current for $R_i = 110$ Ω-cm and for different values of the maximal relative membrane conductances η $N_{Na} = N = 100$ Uniform membrane

alent transformation described here cannot be used

In the case of the uniform membrane the frequency–current relationship is much steeper for η=0 5 and for η=0 the cell hardly fires at all The membrane currents become too small compared to the membrane capacitance and the axial currents to sustain firing when η is reduced first in the initial segment as seen by the reduction of the firing at the lower part of the curve for η=0 5 and then for the soma also as seen for η=0 2

In the case of a passive soma the reduction of η from 1 (broken line) to 0 5 (continuous line) does not change the frequency–current relationship much for I ≤10 μA/cm² as seen from Fig 11 For larger values of the injected current i the firing frequency f decreases when i is increased (filled circles) In the initial segment however f does not decrease (open circles) The firing frequency is taken as the number of spikes generated in one second A spike is defined to have occurred if a certain threshold value of the membrane potential is reached in this case 50 mV above the potassium equilibrium potential E_K is used As the filled circles show the firing frequency in the axon we see from Fig 11 that an increasing number of spikes is not transmitted from the initial segment and down the axon when i is increased This effect is not of course restricted to values of η less than one but it was not seen in the other cases studied here for the current ranges shown in the figures It was however observed for η=1 and $R_i = 110$ Ω-cm shown by the lower curve in Fig 6 when i was increased above 5 μA/cm² For smaller values of i all the spikes were transmitted as for η=0.5 when i is less than 10 μA/cm²

When η=0 2 the cell did not fire at all The reason is the relatively heavier loading of the spike generating compartment by the axial current and membrane resistance as for the case of the uniform membrane

CONCLUSION AND DISCUSSION

The results presented here show that the existence of a finite number of two-state pores can significantly influence the firing behaviour of nerve cells The number of pores in the initial segment is an important parameter For the numbers used here 100 and 1 000 the effects upon the firing behaviour were highly significant for the case of a passive soma but only for N =N =100 for the case of a uniform membrane with the same pore density all over the cell In this latter case the firing behaviour for $N_{Na} = N = 1\,000$ was very close to that of a non-stochastic model This model predicts a non linear frequency–current relationship quite similar to the curves shown by Fig 3 both in the case of a passive soma and of a uniform membrane

In spite of the highly non-li behaviour of the

number of sodium and potassium pores. As shown earlier for the uniform membrane the frequency current relationship is approximately the same for the two different pore systems taken separately (for pore numbers ≥1000), and their combined effect gives only a slightly higher firing frequency (Skaugen & Walløe 1979). For smaller pore numbers the firing behaviour is relatively more dominated by the potassium pore system. As the existence of a two-state sodium pore system is fairly certain (Conti & al. 1976) while this is not known for the potassium pore system where the pore densities also are very uncertain we could have calculated maximum effect of a finite pore number by assuming an infinite number of potassium pores. This situation would be approximated in any case where the membrane electrical noise is dominated by fluctuations of the sodium conductance as would be the case if either the potassium pore density larger than the sodium pore density or the potassium pores do not operate on an on–off basis. The overall firing frequency would then be somewhat smaller than shown in the figures for $N_{Na} = N_K = 100$ and only slightly smaller than shown for $N_{Na} = N_K = 1000$. See also the discussion at the end of the former Papers (Skaugen 1980).

In the former papers effects of other types of electrical noise in the nerve membrane than conductance fluctuations were also briefly discussed. As pointed out there any additional noise would increase the fluctuations of the membrane potential and thus increase the effects studied here. The effects of gating currents were studied in more detail and it was shown that these effects were proportional to the relative rate constants ϕ. Even if gating currents are important in large nerve fibres it thus seems that in the small nerve cells of interest here where the rate constants are much smaller than in large nerve fibres the effects of gating currents would be small.

For the small values of ϕ shown in Table 1 the time course of the action potential in small nerve cells may seem to be excessively slow. But it should be noted that the results are obtained for a temperature of 6.3°C, and at higher temperatures all processes in the nerve membrane will go faster.

The pore density and the dimensions of the initial segment determine the axial resistance and the number of pores in this region, and these parameters were found important for the firing behaviour. By means of the equivalent transformations given by Eq. (4) the models presented here can be used for any pore density. Fig. 1 shows the relation between pore density ρ and the diameter d_i of the initial segment for models A, B and C in Table 1 under these transformations when $R_i = 110$ Ω-cm. The conductances of single pores are assumed equal to those in squid ($u = 1$). The relations are shown both for $N_{Na} = N_K = 1000$ (continuous lines) and for $N_{Na} = N_K = 100$ (broken lines). Along these lines the firing behaviour does not change except that the mean firing frequencies and the injected current shown in the figures are multiplied by ρ/500 pores/μm^2.

The model corresponding to the curve for $R_i = 220$ Ω-cm in Fig. 4 lies between curves B and C in Fig. 12. This will be the uppermost curve where a linear frequency–current relationship was found in the simulations. But from results obtained from the membrane model we suspect model C with for instance 10000 pores to give a frequency–current relationship significantly different from the non-stochastic case. This model will be represented by a curve slightly above the continuous curve marked B in Fig. 1.

The circles represent measured pore densities in different nerve fibres where the range in fibre diameters is indicated by the vertical bars (Colquhoun et al. 1977). The dotted curve is an approximate fit to these data with the pore density for the squid giant axon included ($\rho_{Na} = 500$ pores/μm^2, $d = 500$ μm to 900 μm (Keynes & Rojas 1974)). This lies outside the region shown by the figure.

It is seen that all the types of fibres shown here include diameters where the effects due to a finite pore number are significant. The garfish olfactory nerve corresponds for instance to the curve for $R_i = 550$ Ω-cm in Fig. 5. Even if the pore densities at the site for spike generation are higher than in the axon the figure indicates that some effects are expected for small fibre diameters. Even for pore densities around 100 pores/μm^2 the diameter need not be smaller than 0.2 μm. In general we may conclude that if the diameter at the site for spike generation is less than 0.5 μm to 1 μm, and the length larger than 0.5 space constants, effects due to a finite pore number may be suspected.

I would like to thank Lars Walløe for stimulating discussions, Daniel Kernell and Arild Njå for comments on the manuscript, Sveinung Rekan for running some of the computer programs, and Torlaid Isaachsen for drawing the figures. Part of the work was done at the University

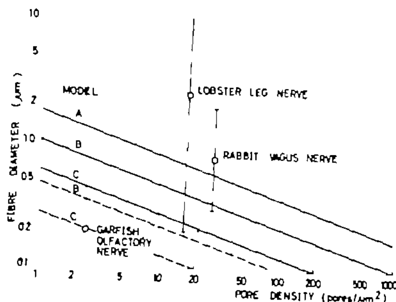


Fig 12 Relation between the diameter of the initial segment and its pore density for some of the cell models used under the equivalent transformations. Continuous lines for $N_{Na}=N_K=1000$ and broken lines for $N_{Na}=N_K=100$. Circles show measured sodium pore densities in different nerve fibres with range of fibre diameters indicated by vertical bars. The length L of the initial segment in units of the space constant λ is 0 18 0 41 and 0 91 respectively for the continuous lines A B and C. Dotted curve is a fit to experimental data, see text

$N_{Na}=N_K=1000$. Whether burst firing would occur for larger pore numbers for the passive soma remains to be investigated but the behaviour of the membrane model strongly suggests that it would be seen at sufficiently large numbers (>10 000)

Small nerve fibres seem to have smaller pore densities than larger fibres (Jack 1975). This does not change the model equations if the rate constants are reduced correspondingly. But a reduction of the pore density relative to the rate constants seems to be very detrimental for the cell's ability to fire as shown by Figs 10 and 11. This is in contrast to the membrane model where a reduction of pore densities alone (not changing rate "constants") down to 0 2 did not stop the firing (Skaugen 1980). This difference is mainly due to the axial currents which are present in the cell model. These depend upon the internal resistance and are not changed when the pore densities are changed. The initial segment is therefore more and more effectively short-circuited to the cell body when the pore densities are reduced. This can be counterbalanced by a corresponding increase of the internal resistance which corresponds to a longer and thinner nerve cell when R_i is kept constant. But it is felt that these difficulties with a reduction of pore densities relative to the rate constant (ϱ/ϕ) do strengthen the results of the discussion in an earlier paper where it was shown that if the H–H equations are used to describe impulse conduction in small unmyelinated nerve fibres the few available experimental data suggest that ϱ/ϕ is constant or even increases when the fibre diameter decrease (Skaugen 1980)

It is now a question how well the nerve cell models presented here represent real nerve cells. As pointed out before nerve cells seem to have pore densities which decrease from the initial segment and towards the dendrites. They would thus lie between the two cases investigated here. It is expected that the effect of reducing the pore density in the soma is to move the steep part of the frequency–current relationship in the uniform membrane case towards larger currents because this part is due to firing in the soma. For a sufficiently small pore density in the soma this relationship would therefore be almost identical to that of the passive soma for reasonable values of the injected current

We have here assumed an equal density of sodium and potassium conducting pores and that both pore systems operate on an on–off basis. The firing behaviour of the model is not however very sensitive to the numerical rel[illegible] between the

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Decreased release of somatostatin into the portal vein following electrical vagal stimulation in the cat

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Electrical vagal stimulations were performed in anesthetized cats, in which the spleen and the intestine below the duodenum had been removed. The cats were provided with acute antral pouches which were perfused during the experiments. Insulin, glucagon, gastrin and somatostatin were measured in portal ein blood (consisting of blood from the stomach and the pancreas). The antral perfusates were analyzed for gastrin and somatostatin. Electrical vagal stimulation increased the release of insulin and glucagon into the portal vein. Gastrin was elevated in antral perfusates and portal vein blood whereas somatostatin increased in the antral perfusates, but was reduced in portal vein blood W assume that antral somatostatin, like gastrin, is released into the blood The finding that portal vein somatostatin w decreased following vagal stimulation indicates that the release of somatostatin from another large source of somatostatin, presumably the fundic region of the stomach or the pancreas was decreased by vagal activation

Key ords Vagal nerve insulin glucagon, gastrin and somatostatin

Several peptide hormones are located in the endocrine cells of the pancreas and of the mucosa of the gastrointestinal tract e.g. insulin glucagon and somatostatin containing cells in the pancreas, gastrin containing G-cells in the antral mucosa and somatostatin containing D-cells along the entire GI tract The release of these peptides is partly under nervous control Accordingly in dogs the output of insulin, glucagon and gastrin increases after sham feeding or electrical vagal stimulation (Frohman Ezdinli & Javid 1967 Kaneto Kosaka & Nakao 1967 Kaneto Miki & Kosaka 1974 Lanciault et al. 1973, Becker Reeder & Thompson 1974 Smith et al. 1975 Nilsson et al 1972 Tepperman Walsh & Preshaw 1972, Nilsson & Uvnas-Wallensten 1974 Nilsson & Uvnäs-Wallensten 1977). In cats electrical vagal stimulation enhances the release of insulin, glucagon and gastrin into the portal vein (Uvnäs-Wallensten & Nilsson 1977 Uvnäs-Wallensten ... & Nilsson 1976) and also the release of gastrin and somatostatin into antral perfusates (Uvnäs-Wallensten 1977 Uvnäs-Wallensten Efendic & Luft 1977)

In the present study we have investigated the effect of electrical vagal stimulation on somatostatin release in cats in vivo Somatostatin was determined in the portal blood and in the antral perfusate

MATERIAL AND METHODS

Experimental procedures

The cats were anesthetized with chloralose and urethane (50 and 100 mg/kg). The trachea was cannulated and the animals breathed spontaneously Blood pressure was continuously measured in femoral artery

The vagal nerves were isolated and cut in the cervical region. The distal ends were stimulated electrically at 10 V 2 ms duration and 5–10 Hz during 5 min

T allow collection of portal blood the spleen and the

Presented at the 1978 Annual Meeting of the European Association for the Study of Diabetes, Zagreb Yugoslavia

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Laboratory of Physiology Oxford It is a pleasure to thank Julian J B Jack and David Whitteridge for their kind hospitality The work was upported by The Norwegian Research Counci for Science and the Humanities

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	30		40
			1 200
	1 000	1 200	
			800
	1 900	1 700	
			1 400
	3 000	2 000	
			400
	200	250	
			600
	0	0	
			650
	200	150	
			100
	0	0	
			90
	0	0	

bodies, produced by ourselves, were used at a final dilution of 1 56000. Crossreactivity of the antibody was less than 0 01% with insulin, glucagon, substance P LH RH vasopressin and oxytocin. The antigenic specificity of the antibodies has been determined using somatostatin analogues (Arimura et al. 1978) Phosphate buffer (0 4 M pH 7 4) containing 1% bovine serum albumin was used as diluent for all components in this RIA. The incubations were conducted for 48 h at 4°C. Separation of bound from free tracer as accomplished with dextrane-coated charcoal (Arimura et al 1973) Somatostatin in plasma was measured after acetone extraction (Arimura et al. 1978). 90-100% of synthetic somatostatin added to plasma was recovered after the full extraction procedure Dilution of both the extracted plasma samples and antral perfusates demonstrated parallel binding to that of the synthetic standard The sensitivity of the assay was 5 pg/ml (Efendic et al 1978)

RESULTS

Hormone release into the portal ein

Somatostatin in portal vein blood was measured in 8 cats before and during electrical vagal stimulation. In 3 of the expts. the antrum was perfused with 0 1 M HCl (pH~1.2) and in 5 expts. with 0 15 MNaCl (pH~6 5) Before the stimulation somatostatin averaged 650 pg/min (range 115–1 500). It decreased within 5 min of stimulation (Table 1 Fig. 1) and then reached prestimulatory values within 10–20 min after its cessation The same pattern was observed irrespective of whether the stimulations were performed with 5 or 10 Hz, or if the antrum was perfused with 0 15 M NaCl or 0 1 M HCl

Portal vein insulin glucagon and gastrin levels were measured in 2 of the expts (Fig 1) During vagal stimulation the output of these hormones reached a peak within 10 min. The levels then decreased and reached the basal ones within 10–20 min.

Hormone release into the antral perf sate

During prestimulatory conditions considerable amounts of somatostatin were released into the antral lumen provided the perfusates had a low pH (exp 1–3 Table 1) On the other hand when the antra were perfused with 0 15 M NaCl only small amounts of somatostatin were released in out of 5 expts. (exp 4 and 6 Table 1).

When the vagal nerves were stimulated, a clear increase in somatostatin output occurred in the 3 expts in which the antrum was perfused with 0 1 M HCl During perfusion with NaCl a slight increase in somatostatin was observed in the expts. in which measurable amounts of somatostatin were released under prestimulatory conditions (Table 1) A typical experiment, demonstrating the effect of vagal stimulation on the release of somatostatin and gastrin into the portal vein and into the antral lumen is shown in Fig. ..

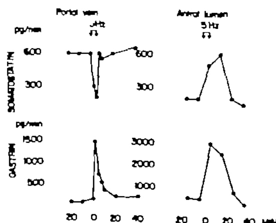


Fig 2 Release of somatostatin and gastrin into the portal vein and antral lumen following electrical vagal stimulation (5 Hz). Note that somatostatin output into the portal vein decreases whereas slight increase is seen in antral perfusates (Expt. 6 from Table 1)

Table 1 *Release of somatostatin into the portal vein (p) and into antral perfusates (a) following electrical vagal stimulation at 5 or 10 H during antral perfusion with 0 1 M HCl and 0 15 M NaCl*

Exp no	Exp conditions		Time in minutes −20		−10		0	2	5	7	10
1	10 Hz	p	1 500		1 500		1 400	200	700	1 000	1 500
	HCl	a		1 200		1 000			1 400		
2	10 Hz	p	600		800		800	200	550	1 200	1 000
	HCl	a		000		2 000			2 500		
3	10 Hz	p	1 200		1 300		1 400	1 200	800	400	
	HCl	a		1 500		1 500			7 000		
4	10 Hz	p	400		350		400	150	100	200	
	NaCl	a		200		250			500		
5	10 Hz	p	600		600		650	250	200	250	300
	NaCl	a		0		0			0		
6	5 Hz	p	600		600		600	300	200	600	350
	NaCl	a		200		200			500		
7	5 Hz	p	150		80		120	40	50	60	70
	NaCl	a		0		0			0		
8	5 Hz	p	120				110	10	20	30	100
	NaCl	a		0		0			0		

intestine below the duodenum were removed a catheter inserted from the blind end of the superior mesenteric vein and the portal vein tied close to the liver. Thus the portal vein conveyed blood from the stomach the pancreas and the duodenum backwards out of the cat. Before entering the cat again the blood passed a dropchamber for recording of the blood flow. The cats were heparinized to avoid clotting of the blood. The preparation has bee described in detail in a previous publication (Uvnäs-Wal lensten Uvnäs & Nilsson 1976)

Blood samples (5 ml) were collected at −20 −10, 0, 2 5 7 10 20 and 40 min. Trasylol (10 %) was added to the samples that were immediately put on ice. After cen trifugation plasma was taken off and frozen.

To allow determination of the intra-antral secretion o gastrin and somatostatin acute antral pouches wer formed by tying ligatures round the pylorus and the an trum-corpus border. The antrum was then perfused wi 0 1 M HCl or 0 15 M NaCl (38°) at a rate of 1 ml/min. Th perfusate entered the antrum via a catheter introduce into the pylorus and left it via a small cannula inserte into the antral wall. For further details of the method se Uvnas-Wallensten (1977)

The antral perfusates were collected in tubes sur rounded by ice during 10-min periods. pH was adjusted t 7 and the samples boiled for 10 min before being frozen The rate of release into the circulation and into the antra lumen of the different peptides was calculated by multiply ng hormone concentrations and portal plasma flow an the flow rate of the antral perfusate respectively

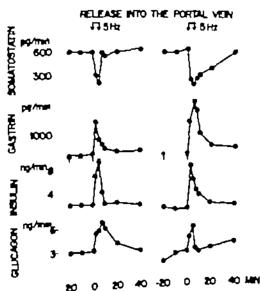


Fig. 1 Release of somatostatin gastrin insulin and glucagon into the portal vein following electrical vagal stimulation (5 Hz). Note that somatostatin output decreases whereas the release of gastrin insulin and glucagon increases. (Expts 6 and 5 from Table 1)

Radioimm noa sa

Insulin was determined by a double-antibody radioim munoassay (Hales & Randle 1963) using insulin reagen kits (Radiochemical Centre Amersham). Glucagon wa assayed using an antibody specific for pancreatic glucago (30 K) (Aguilar Parada Eisentraut & Unger 1969). Gastri was determined by the method described by Nilsso (1975). Somatostatin was measured with radioimmunoas say with the following characteristics. Tyr-somatostati was labelled with J^{125} by the lactoperoxidase method an separated on a carboxymethyl-cellulose column. The sa

This work was supported by the Swedish Medical Research Council (grant No B-76-19X-04540-03) the Nordic Insulin Foundation (Gentofte Denmark) and the Swedish Diabetic Association

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DISCUSSION

The present study demonstrates that electrical stimulation of the vagal nerves was accompanied by a decrease in the release of somatostatin into the portal vein Simultaneously in agreement with previous findings vagal stimulation enhanced the release of insulin glucagon and gastrin into the portal vein (Frohman Ezdinly & Javid 1967 Kaneto Kosaka & Nakao 1967 Kaneto Miki & Kosaka 1974 Lanciault et al 1973 Becker Reeder & Thompson 1974 Smith et al 1975 Uvnäs-Wallensten & Nilsson 1977 Uvnäs Wallensten 1976 Uvnäs Wallensten Uvnäs & Nilsson 1976) and of somatostatin and gastrin into the antral lumen (Uvnäs-Wallensten 1977 Uvnäs-Wallensten Efendic & Luft 1977) We have previously demonstrated that the intraluminal release of gastrin and of somatostatin from the antrum and duodenum in cats is pH dependent (Uvnäs-Wallensten 1977 Uvnäs Wallensten Efendic & Luft 1978) Thus during perfusion of antral or duodenal pouches with neutral or slightly alkaline solutions electrical stimulation caused a release of gastrin into the lumen whereas no or minute amounts of somatostatin appeared in the perfusates During perfusion with 0 1 M HCl on the other hand clearly reduced amounts of gastrin and large amounts of somatostatin could be demonstrated in the perfusates The latter observation was confirmed in the present study Here when the pH of the antral perfusate was kept alkaline when vagal stimulations were performed as expected only minor amounts of somatostatin but large amounts of gastrin were released (Table 1 and Fig. 2) During perfusion with 0 1 M HCl on the other hand substantial quantities of somatostatin appeared in the perfusates

Electrical vagal stimulation releases gastrin both into the lumen and into the circulation (Uvnas Wallensten 1977) It is reasonable to assume that antral somatostatin in addition to being released into the antral lumen is also released into the portal vein following vagal stimulation at low intraantral pH just as electrical vagal stimulation causes a bidirectional release of gastrin (Fig 2) Studies performed by Gustavsson & Lundqvist 1978 and Schusdziarra et al 1978 showing that instillation of acid into the antral lumen enhances the levels of somatostatin in the portal vein confirm this assumption

But since portal somatostatin levels in fact decrease in response to electrical vagal stimulation the lowering of somatostatin levels in the portal vei must be caused by a diminished release of somato statin from another larger source of somatostati thus masking a release of somatostatin from th antrum

One possibility could be that the release of par creatic somatostatin is decreased by vagal stimul tion In favour of this idea is the fact that acety choline and β-endorphine inhibit somatostatin r lease from the perfused pancreas of the dog (Sam et al 1977 Ipp Dobbs & Unger 1976)

Another possible origin of the decreased som tostatin output could be the fundic region the stomach since according to Schusdziarra et a (1978) the output of somatostatin from the antr and fundic regions of the stomach do not alwa parallel each other

From a functional point of view this latter al seems attractive Vagal stimulation is a pote stimulator of gastric acid secretion and somatost tin is an efficient inhibitor of gastric acid secretio

A decreased release of the fundic somatostat might therefore contribute to the stimulatory effe on gastric acid secretion induced by vagal activ tion Whether the postulated inhibitory action somatostatin is exerted locally or via the circulati or by both mechanisms remains to be established

Since it would be impossible to sample pure pa creatic or fundic blood in cats without severe inte ference with the rate of blood flow and there peptide concentrations the question as to the ma source of portal vein somatostatin content has to solved in experiments performed on larg animals

It may be considered justified to attribute t decrease in portal somatostatin levels to an activ tion of vagal cholinergic fibers

However recently the vagal nerve has be shown to contain several types of peptidergic fib including gastrin somatostatin VIP substance and enkephalins (Uvnäs-Wallensten et al 19 Uvnas Wallensten Efendic & Luft 1978 Lundbe et al 1978) Therefore it is possible that a vaga induced decrease of portal vein somatostatin leve is mediated via peptidergic fibers This is support by the finding that in the cat neither vagally i duced release of insulin glucagon or gastrin into t portal vein nor the vagally induced release of ga trin and somatostatin into the antral perfusa could be blocked by atropine (Uvnäs Wallenst 1976 Uvnäs-Wallensten & Andersson 1977)

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Effects of phasic and tonic activation on contraction dynamics in smooth muscle

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UVELIUS B & HELLSTRAND P Effects of phasic and tonic activation on contraction dynamics in smooth muscle Acta Physiol Scand 1980 109 399–406. Received 28 Nov 1979 ISSN 0001-6772 Department of Physiology and Biophysics, University of Lund, Sweden.

Responses to isotonic quick releases of rabbit urinary bladder strips and rat portal veins activated by AC-stimulation and K -high medium were studied The AC stimulation was adjusted to give the same tension as the K -contractures. Releases were performed at peak of the contractions (which was attained after 1.5–4.5 AC-stimulation or 2 3 min in K -high solution) The length response consisted of 3 parts. (1) lastic recoil, (2) isotonic transient, (3) steady shortening Shortening velocity was determined at 100 ms after the release as phase (2) had subsided by then Characteristics of the initial elastic response was virtually unaffected by the mode of stimulation for both preparations. V_{max} was significantly higher for the AC-stimulations than for the K -contractures (bladder 0.37 l/ vs. 0.26 l/s, portal vein 0.46 l/ vs. 0.33 l/s). By means of computer analysis fast xponential shortening component comprising the larger part of phase (2) could be separated from the subsequent slower shortening (see further in Hellstrand & Johansson 1979) I each preparation the amplitude of this exponential was the same for both modes of activation. The time constant was, however smaller for the AC-stimulated preparations. Our results thus indicate that in response to sudden decrease in force the initial elastic recoil is the same whereas the rate of transition to steady shortening, and the steady shortening velocity itself are lower for preparations activated by K -high medium compared to AC-stimulation.

Key ords Urinary bladder portal vein, series lasticity force–velocity relation, mechanical transients, electrical stimulation, K -contraction

The length response to a step change of force in a muscle consists of an initial elastic recoil, followed by a phase of slower active shortening (e.g. Jewell & Wilkie 1958) In skeletal muscle it has been shown that the second phase of shortening is influenced in its earliest part by phenomena re sembling viscoelastic relaxation of complicated form (Podolsky 1960 Civan & Podolsky 1966, Huxley 1974) Corresponding phenomena are seen in the force response to a length step and interpre tations of these effects are attempted in terms of the cross-bridge mechanism of contraction (Podolsky Nolan & Zaveler 1969 Huxley & Simmons 1971 White & Thorson 1973 Abbot & Steiger 1977). Recently it has been demonstrated that phenomena of somewhat similar appearance are encountered in smooth muscle although their time course is much slower and it cannot yet be regarded as certain that they correspond to those in skeletal muscle (Johansson, Hellstrand & Uvelius 1978 Hellstrand & Johansson 1979 Mulvany 1979) One consequence of the presence of the viscoelastic properties is that the velocity of shortening in the second phase of the length response is greatly dependent on time after the release In order to specify the force velocity relation it is thus ecessary to measure shortening elocity at a fixed point in time when the viscoelastic phenomena have essentially subsided (Hellstrand & Johansson 1979)

The immediate series elastic recoil in smooth muscle is several times larger than what is found in skeletal muscle (e.g. Bressler & Clinch 1974 Ford Huxley & Simmons 1977) It is unlikely that major part of the responses is to be scribed to recoil of the individual cross-bridges, as is proposed to be the case in skeletal muscle However a small por

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pling frequency of 1 kHz and analysed by computer program that performed fit of the length record excluding the elastic recoil, to double exponential function of the form

$$L_0 - L \quad A_1[1-\exp(\quad t/\tau\,)] \quad A_2\,[1 \quad \exp(-t/\tau\,)] \tag{1}$$

Each exponential component is thus characterized by its amplitude (A) and its time constant ( ). The elastic recoil was computed by back extrapolation of the fitted curve to a first intersection with the recoil record (cf Fig 1) The amplitude of the force step was also calculated by the computer The fit to the length record was performed within chosen time interval beginning about 8 ms after the force step and extending until 500 ms after the step Occasionally the starting point had to be changed due to irregularities in the record or prominent oscillations, particularly at low afterload and for the portal vein records (where the elastic recoils are greater).

Apart from the elastic recoils and the parameters of the exponentials the program calculates, by differentiation, the shortening velocity at any desired point in time within the interval In this work all velocities are measured at 100 ms after the release at which time the first exponential component (time constant 20–40 ms) has essentially subsided The velocities (V) and afterloads (P) were fitted by a computer program to the Hill (1938) equation:

$$V = b(P \quad P)/(P + \ )\tag{2}$$

The relation characterized by the constants a, b and P (intercept on force axis) In accord with common usage the dimensionless quantity a/P is given here. The maximal shortening velocity (at zero load) is obtained as $V_{max} = bP_0/a$

The elastic properties of smooth muscle are well described by single exponential force–extension relation. The release data were fitted to logarithmic form of the relation

$$\Delta L/L \quad \frac{1}{k} \quad \ln\frac{P \quad B}{A} \tag{3}$$

where A, B and k are constants and L is the initial muscle length

The general procedure of the expts was as follows. After dissection and mounting at preload of <0.2 mN (bladder) or <0 8 mN (portal vein), the muscle was allowed to recover for at least 45 min in tris-buffered Krebs solution of the following composition in mM. NaCl 1?, KCl 4 8 $MgCl_2$ 1 2, $CaCl_2$ 2 5 glucose 11.5 $CaNa_2$-versenate 0 026 and tris-(hydroxymethyl) aminomethane (Trizma Base Sigma Chemical C) 23 The solution was titrated with HCl to pH of 7 4 at 37°C and was bubbled with O_2 The osmolality was about 290 mosm/kg H_2O After the equilibration period the bath was exchanged for one containing solution where 100 mM of NaCl had been replaced with KCl In this K -high solution contracture develops which usually consists of an initial peak, followed by partial relaxation and second slower rising phase, reaching its maximum in about minute (Sigurdsson & Uvelius 1977) After repeated exposures to K -high and normal solution at rigid time schedule (2 3 min contraction 5 min relaxation), highly reproducible responses were obtained Isotonic releases were then performed when the second phase of the isometric responses had reached its maximum. Afterloads were varied in a random fashion. After all releases had been recorded the muscle was kept in normal solution and electrical stimulation (25–100 Hz, 2–4 V duration 2 5) at 1–1 min intervals, was begun. Usually the maximal tension response to AC stimulation was somewhat greater than that to K high solution. To enable comparisons of shortening velocities at similar degree of activation of the muscle the stimulation parameters (frequency and amplitude) were adjusted to give AC responses of similar force as the previously obtained K contractures. The reduction in contractile tension was slight, but it should be emphasized that in this study the muscles were not usually maximally activated during the responses to electrical stimulation. When steady AC responses had been obtained isotonic releases at peak of contraction were performed in similar sequence as for the K contractures.

Statistical comparisons between K and AC responses in the individual muscles were made according to Student' *t*-test for paired values. Amplitudes and time constants of the ponental component in the total material were grouped according to P/P into classes with width of 0.2. Comparisons of K and AC data between corresponding classes were made by Student *t*-test for unpaired values. Significance levels are indicated as. $P<0.05$ $^{**}P<0.01$ ** $P<0.001$

RESULTS

Fig. 1 shows computer displays of force and length records in a portal vein subjected to similar force steps in a K -contracture (K) and an AC-electrically stimulated contraction (AC) Two different time intervals of the same releases are shown (100 ms and 500 ms) Broken line shows a computer fit according to a double exponential model (see Methods). Delineation of the initial elastic length response is shown by the intersection of the fitted line with the length record The rest of the shortening response is treated as sum of two exponentials with time constants 37 ms and 0 61 s (K) and 29 ms and 0.33 s (AC) respectively I the following the responses are treated in this order (1) The initial lastic recoil (SE) (2) the shortening velocity at 100 ms after the release (V) and (3) the amplitudes and time constants of the exponential components of shortening.

1 *Series elasticity*

The initial (series elastic) recoil for different after loads were determined in the manner described above Fig. shows the results for one bladder preparation. Open circles show elastic recoil for

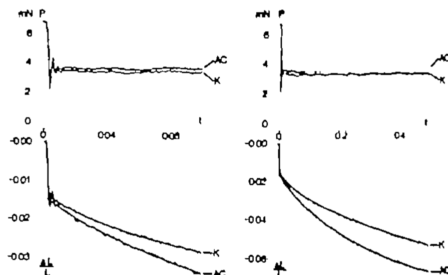


Fig 1 Display of digitized record and curve fits of isotonic quick releases on a portal vein during AC stimulated contraction (AC) and K -induced contracture (K) Left and right panels show same releases on different time scales In each panel top records shows force and bottom records length change (shortening downward) Broken line shows fit to length record according to double exponential model eq (1)

tion of the elastic response in urinary bladder smooth muscle seems to be dependent on the state of activation of the contractile system and may be associated with cross-bridge recoil (Hellstrand & Johansson 1979)

It has repeatedly been suggested that shortening velocity in smooth muscle is lower if the muscle is activated by K high medium rather than by electrical stimulation (Hardung & Laszt 1966 Hellstrand & Johansson 1975 Murphy 1976) Whether this is due to special effects of the K high medium or to differences between "tonic and phasic activity appears not to be known Detailed comparisons in individual muscles have not been reported We considered that this phenomenon might contribute to the understanding of the dynamic properties of smooth muscle In the present study series elasticity force-velocity relations and isotonic transient responses have been characterized in smooth muscle from rabbit urinary bladder and rat portal vein Contractions were elicited by K high solution and by alternating current (AC) electrical stimulation Care was taken to ensure equal isometric tension in the same muscle for both kinds of activation Isotonic quick releases were performed at peak of the contractions

The reason for the use of both the rabbit bladder and the rat portal vein is that in addition to being from different species they represent different types of smooth muscle The portal vein is spontaneously active whereas the bladder is much mor[e] controlled by its rich autonomic nerve supply Mechanical behaviour common to these prepara[-]tions can therefore with greater certainty be con[-]sidered relevant for mammalian smooth muscle [in] general A quantitative study of isotonic transfer responses to quick release in bladder muscle wa[s] done by Hellstrand & Johansson 1979 Here w[e] report such measurements on the portal vein a[s] well Some preliminary results have been presente[d] earlier (Uvelius & Hellstrand 1980)

METHODS

Preparations of smooth muscle from rabbit urinary blad[-]der and rat portal vein were dissected as described b[y] Uvelius (1976) and e g Hellstrand & Johansson (1979) Each muscle strip had a length of 3-7 mm and [was] mounted in the apparatus described by Sjölin Hellstran[d] & Clementz (1978) and by Hellstrand & Johansson (1979) This apparatus allows force steps from isometric cond[i]tions to a constant afterload to be accomplished in les[s] than ms (cf Fig 1) The muscle is mounted horizontally in a thermoregulated trough (1.5 ml) perfused with oxyge[n]ated solution (1 ml/min) and equipped with platinum electrodes for electrical stimulation Three different indi[-]vidually perfused baths can be rapidly exchanged allow[-]ing quick change of solution. The temperature of the bat[h] media was kept at 37°C It was checked that AC stimula[-]tion did not alter temperature in the medium surroundin[g] the muscle Force and length signals were recorded on [a] storage oscilloscope on a paper recorder and on magnetic tape for later computer analysis.

The isotonic release records digitized at a sam[-]

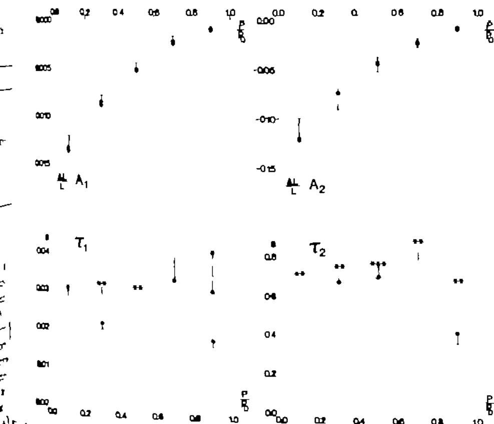


Fig. 4. Amplitudes (A_i) and time constants (τ_i) of the two exponential components ($i=1, 2$) of eq. (1). Bladder strips. Filled circles: AC stimulation, open circles: K -activation. Data grouped into classes according to P/P_o. Each point shows mean ± S.E. n=9–11. Results from 6 expts.

of the portal vein, expressed in muscle lengths per second, is somewhat greater than in bladder. The relative difference in V_{max} between K and AC is about the same in both muscles, 30% lower in K.

Exponential components of shortening

The shortening response after the initial elastic recoil was analyzed according to a model consisting of a combination of two exponential processes, one "fast" and one "slow". The fast component was suggested by Hellstrand & Johansson (1979) to represent a transient phase of cross-bridge rearrangement and change of turnover rate. The slow component was considered to represent the "steady" isotonic response. Some theoretical justification for the choice of an exponential function to describe isotonic shortening exists, but in the actual experiment effects such as temporal variations in activation, possible inactivation due to shortening (cf. results from skeletal muscle by Edman 1976) and non-linear length–tension relations complicate the interpretation of the second phase. In the present context, the analytical description of this phase is used for obtaining the shortening velocity at any desired point in time and as a background for deduction of the fast exponential component (Hellstrand & Johansson 1979).

Fig. 4 shows the amplitude (A_i) and time constants (τ_i) of the "fast" ($i=1$) and "slow" ($i=2$) components obtained from the expts on bladder muscle. A_1 is very similar for the two kinds of activation over the entire range of P/P_o. On the other hand τ_1 is consistently greater in the K responses. The same pattern was seen for A_2 and τ_2, although these parameters are about one order of magnitude greater.

The results from portal vein are shown in Fig. 5. A_1 and τ_1 are rather similar to the bladder data although there is more spread and τ_1 varies with P/P_o in a slightly different way. On the other hand

Table 1 *Mechanical parameters of bladder and portal vein during AC and K activation*

P and V_{max} were determined as the intersections of the force–velocity hyperbola with the force and velocity axes respectively k=stiffness of the elastic element from eq (3) a/P and b=parameters of Hill's eq ( ). Velocity measured 100 ms after release Mean values±S E

	P (mN/mm²)	k	V_{max} (l/s)	a/P	b (l/s)	n
Bladder						
AC	35±4	51±6	0.37±0 05	0.33±0 06	0.11±0 01	6
	ns	ns		ns	ns	
K	34±4	54±3	0 26±0 03	0 40±0 09	0 10±0 0	
Portal vein						
AC	23±?	32±	0 46±0 04	1 4 ±0.59	0.46±0 19	7
	ns	ns		n	ns	
K	?1±1	37±4	0.33±0 0	1 7±0 19	0 55±0 07	

releases performed during K activation and filled circles those during AC stimulation Fits of the elastic length responses to exponential curves are shown The K recoil seems to be somewhat smaller for identical force steps but although this was the most common finding it was not consistent The exponential relation may be characterized by the dimensionless parameter k of eq (3) This is shown in Table 1 Neither for bladder nor portal vein is there any significant difference in k between K and AC although the means for K are greater in both muscles Bladder muscles are generally stiffer than portal veins

? *Force–velocity relations*

Fig 3 shows shortening velocity at 100 ms after release (see Methods) plotted against the afterload P for a bladder strip where the mean isometric tension before release was 11 93 mN in K and 12. mN in AC The curves fitted according to Hill eq (?) intersect with the force axis at points somewhat above these values This was seen in most expt The P values in Table 1 are those obtained from the intersection of the hyperbola with the force axis The shortening velocities in AC are consistently greater than in K for similar afterloads The V_{max} values in the two kinds of contraction are significantly different both for bladder and portal vein (see Table 1) For the same kind of stimulation the V_{max}

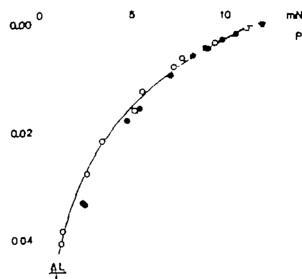


Fig 2 Series elastic recoils of a bladder strip in releases from an isometric tension of 12 mN to different afterloads P Filled circles AC stimulation open circles K activation Exponential fits to both sets of data are shown by broken and full line respectively Constant k of eq (3) is 43 for AC and 50 for K

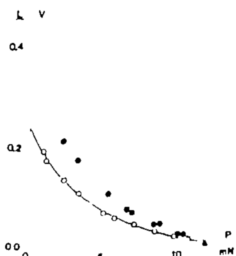


Fig 3 Force velocity relations of bladder strip Velocities measured 100 ms after release Fits of Hill's eq (?) shown. Filled circles broken line AC stimulation Open circles, full line K activation

le & Smith (1973) have reported that cells in smooth muscle, such as rabbit portal-mesenteric vein, swell in solutions where KCl has been substituted for NaCl. They also observed disruptions of thick filaments in muscle fixed after KCl, but suggested that this occurred during the fixation as the contractile response in KCl and K_2SO_4 (which did not cause disruption) were the same. It is possible that cell swelling and filament disruption (if such occurs also in the unfixed muscle in KCl) might be of relevance in explaining the V_{max} differences. There exists however a difference in time course between the K and the AC-contractions as used in this study. While the latter are of short duration (~ 1 s) the K-contractures last for 3 min before the release is made. It is possible that V for a given load decreases with time irrespective of the mode of activation. Such an effect has been described by Lännergren (1978) on slow muscle fibres of Xenopus. Moreover it has been shown for portal vein by Hellstrand & Johansson (1975) and for urinary bladder by U ehara (1979) that V_{max} during a tetanic stimulation decreases more rapidly with time than does the extrapolated P_0. It is known that in portal vein (Hellstrand 1977) the metabolic cost for producing a given mean tension is higher for phasic activity than for K -contractures. Furthermore in K-contractures O_2 consumption in relation to tension development decreases with time (Arner & Hellstrand 1980). It appears possible that an insufficient ATP supply will occur in muscles contracted for longer periods. This might lead to an increase in the life time of attached crossbridges possibly by formation of rigor complexes (Weber & Murray 1973).

For both muscles the amplitude (A) of the fast shortening component is unaffected by the mode of stimulation. Similarly Hellstrand & Johansson (1979) found that A was unaffected by level of activation and by temperature. The fast shortening component was interpreted to represent conformational changes in attached cross-bridges and possibly transient changes in the balance between attachment and detachment of cross-bridges occurring after the force step. It is reasonable to expect that factors which affect the kinetics of cross-bridge turnover will influence primarily the time course of this phase, whereas its amplitude will be determined by the size of the force step.

The rate constant ($1/\tau$) of the fast shortening component is for both preparations about 35% smaller in K -contractures. As mentioned above V_{max} is about 30% lower in K -contractures. Thus the K -contracture affects the rate constant ($1/\tau$) and V_{max} in a comparable way as cooling of the muscle. The similarity in relative difference between $1/\tau$ and V_{max} for the two modes of activation in this study provides further evidence that the fast shortening component is not a passive phenomenon but has relation to the contractile system.

If the level of activation could be held constant long enough after the release the amplitude (A) of the slow exponential component would indicate the amount of shortening needed to reach points on the length-tension curve corresponding to the different afterloads. The time constant ( ) of the component would be determined solely by the force–velocity relation the length–tension relation, and the afterload. In the actual experiment, effects such as decrease with time in the level of activation complicate the interpretation. is larger for K -contractures in both preparations which is to be expected from the change in the force velocity relation. In bladder A_2 is the same for both stimulations whereas in portal vein it is larger for AC-contractions. It seems possible that in portal vein the passive tension which is not negligible might influence the length–tension relation in such a way that the exponential approximation is inapplicable although within a restricted time interval the fits are excellent.

The present study has shown that a homogenously activated smooth muscle can have V_{max} values that differ with the mode of activation despite similar P and elastic properties. The transient isotonic component of shortening has a slower time course in K -contractures, in accordance with the suggestion that it represents relaxation phenomena within the cross-bridge system in response to the force step. The reason for the slower kinetics in K -contractures is not revealed by the present study. Besides effects of the K -high solution as such the longer duration of the contraction before release may be of importance.

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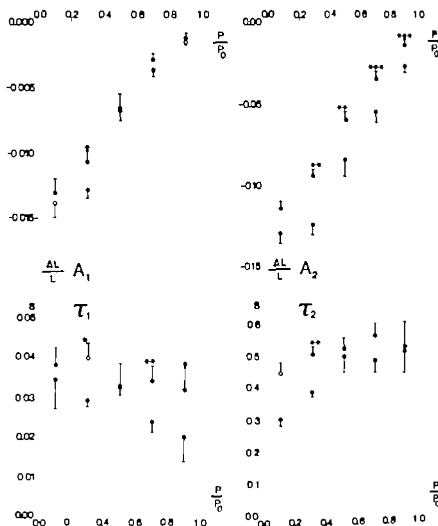


Fig 5 Amplitude (A) and time constant ( ) of the two exponential components (i=1) of eq (1) Portal veins Filled circles AC stimulation open circles K activation Data grouped according to P/P_0. Each point shows mean ± S E n=9–13 Result from 7 expts

there is a consistent difference in A_2 between K and AC expts the amplitudes being numerically greater for the latter τ_2 is greater for K as in urinary bladder

Although the amplitude of the fast exponential component is the same for both modes of stimulation its time course is different as seen by the fact that the rate constant ($1/\tau$) was on the average 25% smaller during K-contracture both in bladders and portal veins

DISCUSSION

The series elastic element (SE) is stiffer in urinary bladder smooth muscle than in portal vein This has previously been shown by Johansson Hellstrand & Uvelius (1978) As is shown in Table 1 there is no significant difference in SE stiffness of either muscle for the two kinds of stimulation although there is a tendency towards a higher k for the K -contractures in both preparations The major portion of the SE undoubtedly resides in passive structures However the results of Hellstrand & Johansson (1979) suggest that part of the recoil (1% or less of the muscle length) occurs in structures associated with the crossbridges If a difference in this fraction of the SE exists between the two kinds of activation it will probably be masked by the large recoil of passive structures and the fact that some variation in P between individual contractions occurred

For the same kind of stimulation the portal veins shorten faster than the bladders For both preparations V_{max} for K -contractures are about 30% lower than for AC stimulations (Table 1) in spite of almost the same tension development Jones Somlyo

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Amylase secretion in response to activation of different autonomic receptors in the rabbit parotid gland

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ASKING, B. & GJÖRSTRUP P Amylase secretion in response to activation of different autonomic receptors in the rabbit parotid gland Acta Physiol Scand 1980 109 407–413 Received 29 Nov 1979 ISSN 0001-6772. Institute of Physiology and Biophysics University of Lund S eden.

The contribution by different autonomic receptors to the amylase secretion from the parotid gland of the anaesthetized rabbit was studied as the response to arious parasympathomimetic and sympathomimetic drugs Amylase secretion by infusions of pilocarpine or parasympathetic nerv stimulation was low but regularly higher in response to pilocarpine than to parasympathetic nerve stimulation. These effects were reduced to the same level by β-adrenoceptor block indicating the presence and for pilocarpine also the release of catecholamines probably from the adrenals Isoprenaline injections produced high amylase secretion, that was blocked by atenolol indicating that predominantly β_1-adrenoceptors were activated Phenylephrine was without amylase secretory effects. By accepting isoprenaline maximum as maximum for ympathetically produced amylase secretion, theoretical frequency-response relationship for amylase secretion by sympathetic nerve stimulation could be calculated ED_{50} w 0.9 Hz. The results indicate that under experimental conditions in viv there are certain differences between the rat and the rabbit parotid glands in the autonomic control not only of fluid but particularly of amylase secretion.

Key ord Rabbit, parotid gland, amylase secretion α- and β-adrenoceptors, muscarinic receptor

Amylase secretion in salivary glands has mainly been studied in the rat parotid gland. In vitro amylase predominantly released on β-adrenoceptor activation, but some amylase is also obtained on stimulation of α-adrenoceptors or muscarinic receptors (see Schramm & Selinger 1975) However in vivo the situation is more complex in that the output of amylase by parasympathetic nerve stimulation can be as great as that by ympathetic activation (Garrett & Thulin 1975 Garrett Thulin & Kidd 1978) Fluid secretion like in most salivary glands (see Schneyer & Emmelin 1974) is predominantly evoked by parasympathetic nerves but a slow sustained flow of saliv can be obtained also on sympathetic nerve stimulation an effect con eyed via both α- and β-adrenoceptors (Garrett & Thulin 1975)

About the rabbit parotid gland les is known but in vivo experiment indicate that parasympathetic nerve stimulation evokes secretion of fluid with content of amylase The amylase secretion seems mainly to be under the control of the sympathetic nerves and in the presence of a parasympathetic salivary flow large amounts of amylase can be released at stimulation frequencies too low to evoke any fluid secretion. The sympathetic fluid secretion is sparse and mainly mediated via α-adrenoceptors (Gjörstrup 1977 1978 1979)

The present experiments set out to more closely investigate to what xtent activation of different autonomic receptors contributes to the secretion of amylase from the rabbit parotid gland. The effects of injections of various autonomic drugs were studied rather than ympathetic nerve stimulation. In this way it was possible to overcome vasoconstriction which particularly in the rabbit salivary glands, tends to severely retard the fluid secretion necessary to transport the amylase (see Gjörstrup 1979).

METHODS

30 rabbits, 1 7–3 4 kg and kept on standard pelleted diet, were used 22 26 h prior to the experiments the animal

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The contribution by different autonomic receptors to the amylase secretion from the parotid gland of the anaesthetized rabbit was studied as the response to various parasympathomimetic and sympathomimetic drugs. Amylase secretion by infusions of pilocarpine or parasympathetic nerve stimulation was low but regularly higher in response to pilocarpine than to parasympathetic nerve stimulation. These effects were reduced to the same level by β-adrenoceptor block indicating the presence and for pilocarpine also the release of catecholamines, probably from the adrenals. Isoprenaline injections produced high amylase secretion, that was blocked by atenolol, indicating that predominantly β_1-adrenoceptors were activated. Phenylephrine was without amylase secretory effects. By accepting isoprenaline maximum as maximum for sympathetically produced amylase secretion, theoretical frequency-response relationship for amylase secretion by sympathetic nerve stimulation could be calculated. ED_{50} was 0.9 Hz. The results indicate that under experimental conditions in vivo there are certain differences between the rat and the rabbit parotid glands in the autonomic control not only of fluid, but particularly of amylase secretion.

Key words: Rabbit, parotid gland, amylase secretion, α- and β-adrenoceptors, muscarinic receptors

Amylase secretion in salivary glands has mainly been studied in the rat parotid gland. In vitro amylase is predominantly released on β-adrenoceptor activation, but some amylase is also obtained on stimulation of α-adrenoceptors or muscarinic receptors (see Schramm & Selinger 1975). However, in vivo the situation is more complex in that the output of amylase by parasympathetic nerve stimulation can be as great as that by sympathetic activation (Garrett & Thulin 1975, Garrett, Thulin & Kidd 1978). Fluid secretion, like in most salivary glands (see Schneyer & Emmelin 1974) is predominantly evoked by parasympathetic nerves but a slow sustained flow of saliva can be obtained also on sympathetic nerve stimulation, an effect conveyed via both α- and β-adrenoceptors (Garrett & Thulin 1975, Thulin 1975).

About the rabbit parotid gland less is known, but previous experiments indicate that parasympathetic nerve stimulation evokes secretion of fluid with low content of amylase. The amylase secretion seems mainly to be under the control of the sympathetic nerves and in the presence of a parasympathetic salivary flow large amounts of amylase can be released at stimulation frequencies too low to evoke any fluid secretion. The sympathetic fluid secretion is sparse and mainly mediated via α-adrenoceptors (Gjörstrup 1977, 1978, 1979).

The present experiments set out to more closely investigate to what extent activation of different autonomic receptors contributes to the secretion of amylase from the rabbit parotid gland. The effects of injections of various autonomic drugs were studied, rather than sympathetic nerve stimulation. In this way it was possible to overcome vasoconstriction, which, particularly in the rabbit salivary glands, tends to severely retard the fluid secretion necessary to transport the amylase (see Gjörstrup 1979).

METHODS

30 rabbits, 1.7–3.4 kg and kept on standard pelleted diet, were used. 22–26 h prior to the experiments the animal

were deprived of food but allowed water ad libitum. In the acute experiments anaesthesia was induced by injecting urethane 1.5–1.7 g/kg into a lateral ear vein; additional doses were given through a cannulated femoral vein. Elimination of the corneal reflex was considered a sign of a satisfactory depth of anaesthesia. A tracheal cannula was inserted. In 5 rabbits the right superior cervical ganglion was removed in pentobarbitone anesthesia (35 mg/kg i.v.) 3 to 4 days prior to the acute experiment. This time allowed for the gland to be reloaded with amylase after the sympathetic degeneration secretion that follows ganglionectomy (Garrett & Thulin 1975*b*).

To register the rate of fluid secretion and to collect saliva for amylase determination the right parotid duct was cannulated as close to its orifice in the mouth as possible. Glass cannulae giving drops of a size of 15 μl for distilled water were used. The drops were recorded on a smoked drum by an ordinate writer manually operated. Parasympathetic fluid secretion was evoked by electrical stimulation (supramaximal intensity, ms duration) of the auriculo-temporal nerve which was dissected as described by Burgen (1964) and Nawrocki (1868). Drugs were injected or infused either i.v. through the femoral vein or i.a. via the lingual artery after ligation of all major branches from the external carotid artery including the facial artery distal to the parotid gland. The blood flow through the submaxillary gland was eliminated by removing the gland. The i.a. route was regularly used for α-blocking drugs to avoid the fall in blood pressure that is particularly marked in the rabbit on i.v. injections (Harvey & Nickerson 1953, Gjörstrup unpublished). In 9 acute expt. adrenalectomy was performed via a mid line abdominal incision. The vascular supply was either ligated and the adrenals were removed or the vessels were clamped leaving the adrenals in situ depending on the access to the gland.

Drugs were injected either to a nonsecreting gland or in the presence of a parasympathetic fluid secretion imitating the resting secretion normally present in the waking animal (Schneyer & Emmelin 1974). As has been shown previously, 50–100 μl/min is a suitable background flow of saliva and was evoked by parasympathetic stimulation at 1.? Hz (Gjörstrup 1979). Saliva for amylase determinations was always collected over 5 min. When drugs were injected while parasympathetic secretion was going on the experimental schedule was as follows. For each dose of a drug to be used a control sample of parasympathetic saliva was first obtained. At the precise end of that period the drug was injected and saliva for a second period of 5 min was collected. To make allowance for dead space in the cannulae and the time for the drug to reach the gland the first drops formed during this period were regularly omitted. To make sure that the effect following the injection of the stimulating drug had worn off another 5 min passed before the procedure was repeated.

The samples of saliva were stored in deep freeze until assayed according to the method described by Dahlqvist (196?). In this method 1 unit of amylase is equivalent to the amount that liberates reducing groups corresponding to 1 micromole of maltose formed each minute at ?°C. Samples of saliva of 50 or 100 μl were used and diluted 100–1000 times before processing.

Amylase obtained in the saliva is expressed as sample concentration of amylase (units/ml) and as output of amylase per min (units/min).

Statistics

Student's *t*-test for paired and unpaired observations was used to test significance. *P* values less than 0.05 were considered significant.

Drugs

Pilocarpine hydrochloride, adrenaline bitartrate, noradrenaline bitartrate, phenylephrine hydrochloride, isoprenaline sulphate, salbutamol (obtained from Allen & Hanburys), atenolol (ICI), dihydroergotamine (Sandoz).

RESULTS

Parasympathetic activation

The parasympathetic saliva that served as a background flow for injections of various sympathomimetic drugs showed an amylase concentration of 433±38 units/ml (mean±S.E., $n=6$). A continuous background flow of saliva of 50–100 μl/min could also be obtained on pilocarpine infusions at ?? μg/kg/min i.v.; however, the amylase concentration in this saliva was higher, being 644±60 units/ml ($P<0.0?$, $n=6$). Extirpation of the superior cervical ganglion 3–4 days prior to pilocarpine infusion further increased the amylase concentration in the saliva: 1001±51 units/ml ($P<0.001$, $n=5$). When pilocarpine 10 μg/kg was injected i.a. and saliva collected at flow rates of 80–100 μl/min the amylase concentration was of the same order as that in response to parasympathetic activation, being 351±25 units/ml ($n=3$). After β_1-adrenoceptor block by atenolol ? mg/kg the amylase concentration in response to parasympathetic nerve stimulation or pilocarpine administration fell to values between ?? and 19 units/ml. Nine attempts were made to study amylase secretion evoked by auriculotemporal nerve stimulation or pilocarpine infusion after adrenalectomy. However, only one experiment was successful: despite accurate and fast surgical procedures the rabbits died in vascular shock within 15–30 min after elimination of the second adrenal. In the successful experiment the amylase concentration produced by auriculotemporal nerve stimulation was 35? units/ml (mean of 9 observations) before and 140 units/ml (mean of 4 observations) after adrenalectomy. The amylase concentration was not further lowered by atenolol ? mg/kg.

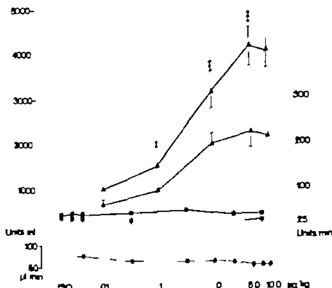


Fig. 1. Amylase secretion in response to various doses of isoprenaline, µg/kg. Injections ere given against background of parasympathetically evoked fluid secretion that as continuous throughout the experiment. The verage secretory rates when samples of saliva were collected is shown (● -●). Each sample of saliva collected after an injection of isoprenaline was preceded by the collection of parasympathetic control sample. Each experiment was begun by collecting 3 control samples. The amylase values are shown separately for those obtained after isoprenaline injections (▲) and for those which are parasympathetic controls (■). The amylase secretion is expressed as concentration in units/ml (—) and as output in units/min (--). Mean ±S.E. from 6 expts. $P<0.05$ $P<0.02$; ** $P<0.001$ vs. preceding parasympathetic controls

Sympathomimetic drugs

Isoprenaline, 10–100 µg/kg did not cause any flow of saliva. However the amylase content in the saliva markedly increased when isoprenaline was injected while parasympathetic secretion 50–100 µl/min was going on. Already doses as low as 0.01 µg/kg were regularly effective and maximal amylase secretion was evoked by doses around 5 µg/kg (Fig. 1). Superimposed isoprenaline injections did not influence the rate of fluid secretion. Atenolol, 2–4 mg/kg, markedly lowered or abolished the secretory response to isoprenaline (Fig. 3). Salbutamol, at high doses only 50–100 µg/kg, injected after atenolol and with parasympathetic secretion present, caused a minor increase in the amylase concentration in the saliva (Fig. 2): the flow rate was unaffected and on its own salbutamol had no fluid secretory effect at 100 µg/kg. Phenylephrine, 30–50 µg/kg, administered under the same experimental conditions as those for salbutamol was completely without effect on amylase or fluid secretion (Fig. 2).

Injections of noradrenaline and adrenaline in the presence of a parasympathetic background secretion caused a dose dependent increase in amylase concentration in the saliva for doses between 0.1–10 µg/kg, without changing the rate of fluid secretion (Fig. 3). On high doses 10–30 µg/kg, a further increase in amylase concentration was parallelled by decrease in flow of saliva, which in fact decreased the output of amylase per min. The reduction in salivary flow rate to doses of noradrenaline and adrenaline above 10 µg/kg persisted almost unaffected after i.a. injections of dihydroergotamine 0.1–1 mg/kg. On their own noradrenaline and adrenaline were without fluid secretory effects.

Sympathetic frequency-response curve

The intense vasoconstriction seen at sympathetic stimulation above 1 Hz has not made it possible to experimentally obtain frequency-amylase response curve for the whole range of sympathetic stimulation frequencies, so far only the relationship between 0.05 and 1 Hz is known (Gjörstrup 1979).

were deprived of food but allowed water ad libitum In the acute experiments anesthesia was induced by injecting urethane 1.5–1 7 g/kg into a lateral ear vein additional doses were given through a cannulated femoral vein Elimination of the corneal reflex was considered a sign of a satisfactory depth of anaesthesia. A tracheal cannula was inserted In 5 rabbits the right superior cervical ganglion was removed in pentobarbitone anesthesia 35 mg/kg i v 3 to 4 days prior to the acute experiment This time allowed for the gland to be reloaded with amylase after the sympathetic degeneration secretion that follows ganglionectomy (Garrett & Thulin 1975b)

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tamol, while phenylephrine was without effect. The results indicate that on sympathetic nerve stimulation amylase secretion is almost exclusively an effect of β_1-adrenoceptor activation. The amylase secretion remaining in response to low doses of isoprenaline after β_1-adrenoceptor block, could equally well have been due to an incomplete block by atenolol, as to activation of a sparce number of β_2-adrenoceptors, indicated by the high doses of salbutamol needed to obtain some amylase. Similarly to the rabbit, the β-adrenoceptors that mediates amylase secretion in the rat parotid gland are mainly of the β_1-type but amylase can also be released by drugs known to act more selectively on the β_2-adrenoceptors (Carlsöö, Danielsson & Henriksson 1978). Previous work on the rabbit parotid gland has indicated activation of β-adrenoceptors as the most efficient adrenergic stimulus of amylase secretion (Yamamoto et al. 1968) however variable flow-rates and a different experimental technique make it difficult to judge amylase output and to compare it to the present findings.

Noradrenaline and adrenaline closely resembled sympathetic nerve stimulation in that doses higher than those causing 50–70 % of the maximal amylase secretion were followed by a reduced fluid secretion, interpreted as an effect of vasoconstriction. It was not possible to further increase the doses of adrenaline or noradrenaline after α-adrenoceptor block without diminishing the fluid secretion to the same extent as seen prior to block indicating, like earlier investigations have done that sympathetically evoked vasoconstriction is difficult to abolish in the rabbit (Harvey & Nickerson 1953, Gjörstrup unpublished).

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The fluid secretory responses when phenylephrine, adrenaline or isoprenaline were injected to the quiescent rabbit parotid gland are in accordance with earlier observations (Gjörstrup 1977

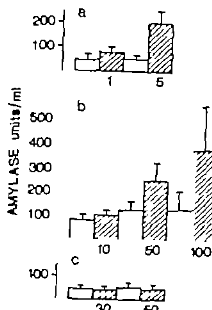


Fig 2 Effects of isoprenaline (a) salbutamol (b), phenylephrine (c) on amylase secretion doses in µg/kg The drugs were injected after β-adrenoceptor block by atenolol 2 mg/kg for salbutamol and phenylephrine and 2–4 mg/kg for isoprenaline The experiments were performed in the presence of a parasympathetic background flow of saliva 50–85 µl/min The observation on isoprenaline are from the same experiments as those presented in Fig 1 Open columns amylase concentration in preceding parasympathetic controls All values are mean ±S E of 5–7 expts

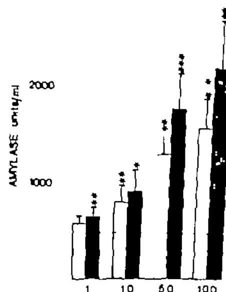


Fig 3 Amylase secretion in response to noradrenaline and adrenaline µg/kg injected in the presence of a parasympathetically evoked fluid secretion 70–90 µl/min The scheme shown in Fig 1 has been followed Open columns noradrenaline black columns, adrenaline $P<0.0$_ $P<0.001$ vs. preceding parasympathetic controls Mean±S.E of 5 expts. Dotted line shows mean amylase concentration in parasympathetic control samples

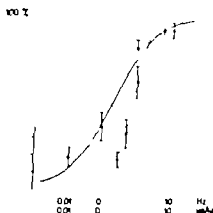


Fig 4 Frequency-response curve for sympathetic nerve stimulation (broken line) and dose response curve for isoprenaline (continuous line). Amylase secretion as the output units/min in response to individual sympathetic nerve stimulations (from Gjörstrup 1979) and to individual isoprenaline injections (mean values shown in Fig 1) have been expressed as percentage of mean maximal amylase output by isoprenaline 5 µg/kg (Fig 1) The percentages have been fitted to a logistic function, for further details see the text Mean ±S E. of 6 expts. with isoprenaline and 7 with sympathetic nerve stimulation

The previously obtained amylase output/min response to sympathetic nerve stimulation was recalculated as net values by subtracting the amylase output in preceding parasympathetic controls and expressed as percentage of the presently obtained mean isoprenaline maximum (Fig. 1) The amylase secretion evoked by the different isoprenaline doses (Fig 1) was recalculated in the same way All values were fitted to a logistic function as deviced by Parker & Waud (1971) the resultant theoretical frequency response curve for sympathetic nerve stimulation and its experimental analogue for isoprenaline are shown in Fig. 4 ED_{50} for sympathetic nerve stimulation was 0.9 Hz and for isoprenaline injections 0 18 µg/kg

DISCUSSION

Noradrenaline adrenaline and isoprenaline injected in the presence of a parasympathetic fluid secretion could all imitate the amylase secretion seen at sympathetic nerve stimulation under similar conditions (Gjorstrup 1979) The effects of isoprenaline were lowered or abolished by atenolol and after β adrenoceptor block only a very sparse increase in amylase secretion was seen on injections of sal

butamol, while phenylephrine was without effect. The results indicate that on sympathetic nerve stimulation amylase secretion is almost exclusively an effect of β_1-adrenoceptor activation. The amylase secretion remaining in response to low doses of isoprenaline after β_1-adrenoceptor block could equally well have been due to an incomplete block by atenolol, as to activation of a sparce number of β_2-adrenoceptors, indicated by the high doses of salbutamol needed to obtain some amylase. Similarly to the rabbit, the β-adrenoceptors that mediate amylase secretion in the rat parotid gland are mainly of the β_1-type but amylase can also be released by drugs known to act more selectively on the β_2-adrenoceptors (Carlsöö, Danielsson & Henriksson 1978). Previous work on the rabbit parotid gland has indicated activation of β-adrenoceptors as the most efficient adrenergic stimulus of amylase secretion (Yamamoto et al. 1968), however variable flow-rates and a different experimental technique make it difficult to judge amylase output and to compare it to the present findings.

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Nordenfelt & Ohlm 1957 Ohlin 1964) which have shown only very sparse if any fluid secretory effects on activation of α or β-adrenoceptors by the doses presently used The same doses of these drugs regularly evoke fluid secretion in the rat parotid gland (Ekström 1973 Ohlin 1964)

The results show that there are certain differences between rats and rabbits in the nervous control of secretion of amylase and fluid from the parotid gland in vivo In the rabbit amylase secretion is practically only an effect of β adrenoceptor activation whereas in the rat the output can be as great at parasympathetic nerve stimulation as at sympathetic Another major difference is that maximal amylase secretion for sympathetic stimuli can be evoked without any accompanying fluid secretion which is the case in the rat Thus the nervous control of the amylase and fluid secretion in the rabbit can experimentally be more separated than in the rat which may prove helpful for studies on the interactions between parasympathetic and sympathetic nerves in their control of salivary amylase and fluid secretion

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Effects of carbachol on isoprenaline evoked amylase release from the rabbit parotid gland in vitro

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ASKING B. & GJORSTRUP P Effects of carbachol on isoprenaline evoked amylase release from the rabbit parotid gland in vitro Acta Physiol Scand 1980 109 415–420 Received 29 Nov 1979 ISSN 0001-6772 Institute of Physiology and Biophysics, University of Lund S eden.

Amylase secretion from dispersed lobules of the parotid gland of the rabbit was studied in response to isoprenaline (10^{-8} 10^{-4} M) and to carbachol (10^{-8} 10^{-4} M). The effects of each agent were investigated alone and in combination at certain concentrations. Isoprenaline produced dose-related amylase secretion with an verage maximum of 688 units/100 mg, at 10^{-6} M The amylase secretion produced by submaximal concentrations of isoprenaline could be further increased by carbachol, already in low concentrations (10^{-8} 10^{-} M), that were subthreshold for amylase secretion. This potentiating effect was seen not only as larger secretion but also as greater depletion of amylase from the tissue. In contrast to what is known from experiments in vivo carbachol in high concentration (10^{-} M), by activating muscarinic receptors only released amylase to the same xtent as that released by isoprenaline. This concentration of carbachol regularly decreased the amylase secretion evoked by isoprenaline and may be regarded as unphysiologically high. The increase in isoprenaline evoked amylase secretion brought about by carbachol in lower concentrations, may be due to an improved transport of amylase, because of secretion of fluid, but could also be caused by augmentation of the β-adrenoceptor mediated effects.

Key words. Rabbit, parotid gland, amylase secretion, β-adrenoceptor activation, carbachol, isoprenaline

Experiments in vitro and in vivo have established that the release of amylase from parotid salivary glands is predominantly due to activation of β-adrenoceptors (see Schramm & Selinger 1976, Asking et al. 1979 Gjorstrup 1979*a* Asking & Gjörstrup 1980) In experiments on the rabbit parotid gland sympathetic nerve stimulation or injections of isoprenaline ere found to greatly increase the content of amylase in a parasympathetically produced saliva, already at frequencies or doses below threshold for fluid secretion (Asking & Gjorstrup 1980 Gjörstrup 1977 Gjörstrup 1979) This importance of the parasympathetic nerves for the production of a salivary flow that can transport sympathetically released amylase along the glandular ducts has recently been shown also in the conscious animal during feeding (Gjörstrup 1979*b*) However it is conceivable that the parasympathetic activity apart from producing fluid may have augmented the β-adrenoceptor mediated amylase secretion. Sympathetically evoked fluid secretion is known to be augmented by parasympathetic activity (see Emmelin 1979) and thus has been demonstrated for the rabbit parotid gland also (Gjörstrup 1977)

In an attempt to btain further knowledge about the promoting action of parasympathetic activity on the sympathetically evoked secretion of amylase the interaction between sympathetic and parasympathetic secretory stimuli was studied in a system that minimizes the intraglandular transport distance for released amylase and thus the dependence of the salivary flow through the glandular duct system, that is necessary in vivo The present experiments describe isoprenaline evoked amylase secretion from dispersed rabbit parotid lobules and how this secretion is influenced by simultaneous carbachol stimulation

METHODS

Parotid glands from 28 rabbits, 1.5–.5 kg, were used. The animals were kept on standard pelleted diet and starved but allowed water 24 h prior to the expts. To remove the

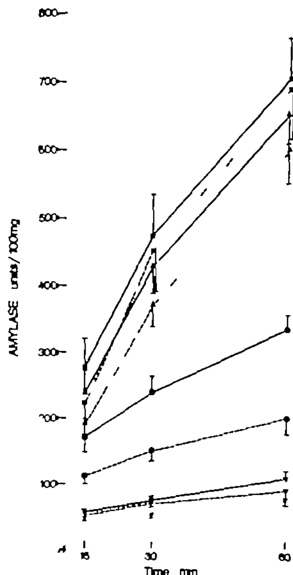


Fig 1 Amylase secretion in response to different doses of isoprenaline alone (broken lines) and in the presence of carbachol in a concentration of 10 M (solid lines) Dotted line represents controls The secretion is expressed as units of amylase released per 100 mg of parotid tis ue Isoprenaline was given in the following concentrations (M) 10 (▼) 10 (●) 10^{-4} (▲) 10 (■) The observations with carbachol alone (10 M) did not differ from controls and have been omitted from the figure to make t plainer They were as follows at the different time 43+6 54±8 77±11 units/100 mg Each point of observation is the mean ±S E of 7 9 expts.

glands the animals were anesthetized with urethane 2 0 g/kg given into a lateral ear vein. A tracheal cannula was inserted The anesthesia made it possible to remove most of the fat and connective tissue from the gland in situ thereby minimizing the time between the removal of the gland and the start of the experiment The glands were transferred to pregassed chilled (4°C) incubation medium which was allowed to slowly reach room temperature during further dissection Instead of using tissue slices fine lobules of a size of 1×2×2–3 mm were cut out by following the normal lobular anatomy of the gland. The procedure is likely to cause less damage to the acini than tissue slicing (Castle et al 1971). Preliminary expts. showed that with this procedure the basal leakage of amylase during subsequent unstimulated incubation was low and that 90 95 % of the original amount of the glandular amylase could be recovered from the tissue after incubation for 1 h

After the final dissection samples of lobules were rapidly weighed and 45–55 mg of tissue was transferred to 25 ml Erlenmeyer flasks containing 10 ml of fresh medium The incubation flasks were placed in a metabolic shaker bath shaking at a frequency of 150 per minute and kept at a temperature of 37°C. After transfer to the bath the tissue was washed three times before stimulating or blocking agents were added Washing was done by decanting medium and adding 10 ml of fresh medium at 5–10 min intervals. The medium was of Krebs-Ringer type and had the following composition (mM). NaCl 120, KCl 4.8 $CaCl_2$ 2 6 $MgCl_2$ 1 5 NaH_2PO_4 1 5 $NaHCO_3$ 25 In addition the medium was fortified with glucose (1 mM) and β hydroxybutyric acid (0 5 mM) The flasks were continuously gassed with a mixture of 95 % O_2 and 5 % CO_2 pH 7 40 After addition of stimulating drugs, samples of 0 1 ml were taken at 15 30 and 60 min for amylase determination Blocking drugs were added 10 min prior to stimulating drugs. All drugs were given in a volume of 0.1 ml

For amylase determination a 0 1 ml sample was transferred to 0 9 ml phosphate buffer and then processed according to Dahlqvist (1962) In this method 1 unit of amylase is equivalent to the amount that liberates reducing groups corresponding to 1 μmol of maltose formed each minute at 25°C Tissue content of amylase was measured as content in supernatants of spun tissue homogenates which were obtained by homogenizing incubated tissue in cold phosphate buffer immediately after the end of an expt

Statistic

Student's *t*-test for paired observation was used *P* values less than 0 05 were considered significant

Drug

Carbachol chloride isoprenaline sulphate propranolol (ICI) Hoechst 9980 (n peridino-etyl-diphenyl-acetamid hydrochloride) All drugs were dissolved in physiological saline solution

Results

Isoprenaline (10 –10 M) produced a dose related increase of amylase secretion that could be further increased by the addition of carbachol in concentrations of 10 –10 M This effect on the isoprenaline evoked amylase secretion was particularly studied at a concentration of carbachol of 10 M a carbachol concentration which was without own amylase secretory effect The additional increase in

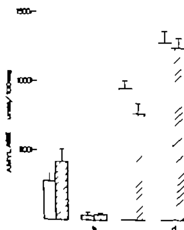


Fig. 2. Amylase depletion from parotid tissue after 60 min incubation with isoprenaline in concentration of 10⁻ M either alone (open columns) or in combination with carbachol at concentration of 10^{-6} M (hatched columns). Amylase secretion and tissue content is expressed as units per 100 mg of parotid tissue. Controls (not shown) released 87±7 units/100 mg after 1.5 h incubation. Amylase release in the presence of agonists: (*a*) amylase release during additional 30 min incubation in absence of agonist following directly after exposure to agonists: (*b*) amylase remaining in tissue at end of incubation. (*c*) total amount of amylase (sum of +*b*+*c*). *d*. Mean ±S.E. from 4 expts.

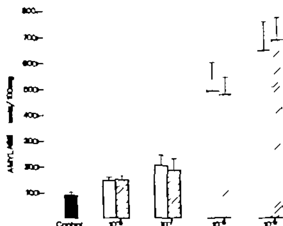


Fig. 3. Amylase secretion in response to 60 min incubation with isoprenaline (10^{-6}–10^{-8} M) alone (open columns) and in combination with carbachol (10^{-6} M, hatched columns). Black column indicate controls. In all cases the atropine-like agent Hoechst 9980 (10^{-6} M) was added 10 min prior to agonists. The secretion is expressed as units released per 100 mg of parotid tissue. Mean ±S.E. from 5 expts.

amylase release that carbachol brought about for each concentration of isoprenaline was maintained at an almost unchanged level throughout the expts. which lasted for one hour (Fig. 1). The concentration of isoprenaline necessary to evoke amylase secretion was around 10^{-8} M which tended to increase the amylase release however significantly only in one series of experiments (Fig. 3, $P<0.05$). Fig. 1 shows that the presence of carbachol (10^{-6} M) regularly caused isoprenaline in this low concentration to release a significant amount of amylase after 30 ($P<0.02$) and after 60 ($P<0.01$) but not after 15 min incubation. The most pronounced effect of the addition of carbachol was seen at an isoprenaline concentration of 10⁻ M: the amylase release by isoprenaline was increased two-fold at all three times of determination ($P<0.001$). When the concentration of isoprenaline was increased to 10^{-6} M, carbachol could still promote an increase in amylase release by an additional 10% measured at 15, 30 and 60 min of incubation ($P<0.02$). The amylase release in response to higher concentrations (10^{-6} M) was found to be maximal for isoprenaline and the amylase secretion was not further increased by the addition of carbachol. The promoting effects of carbachol (10^{-6} M) on isoprenaline (10⁻ M) evoked amylase release was also reflected in the amount of amylase that remained in the tissue after cessation of stimulation. Fig. 2 shows a significantly ($P<0.05$) greater depletion of amylase in the lobules which in the presence of carbachol had secreted more amylase than in those which in the presence of isoprenaline only secreted less. In those experiments the 60 min of incubation with the stimulating agents was followed by an additional incubation period of 30 min in the absence of the drugs before the tissue content of amylase was determined. This was done to allow for any released amylase that could remain in the ducts to diffuse into the medium. For both conditions of incubation the amount of amylase released during the additional 30 min incubation and the total amount of amylase was the same. The promoting effect on the isoprenaline evoked amylase release that carbachol brought about was completely abolished by the atropine-like agent Hoechst 9980 which left the isoprenaline produced amylase secretion unaffected (Fig. 3).

The promoting effects of higher concentrations of carbachol (10⁻ – 10^{-6} M) were studied at a certain concentration of isoprenaline (10⁻ M). Concentrations of carbachol at 10^{-6} and 10^{-6} M produced a

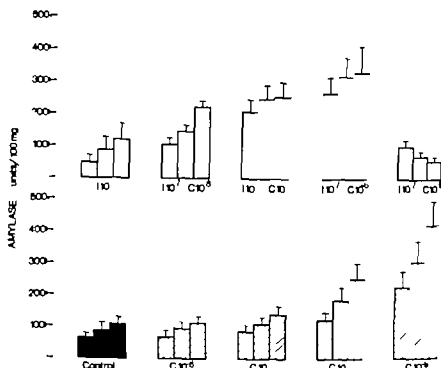


Fig 4 The hatched column show amylase secretion in response to carbachol (C 10 –10 M) The amylase leakage from controls is shown as the black column Above is shown the net amylase secretory effects by isoprenaline (I 10 M) alone and in the presence of carbachol of different concentrations The net amylase secretory effect of isoprenaline has been obtained by subtracting the leakage in control plus the secretory effects by carbachol in paired experiments The three columns represent 15 30 and 60 min after addition of stimulating agents. The observations are the mean ±S E from 5 expt

continuous amylase secretion which was significantly larger than the leakage from controls ($P<0.05$ $P<0.0?$ respectively Fig 4) Carbachol

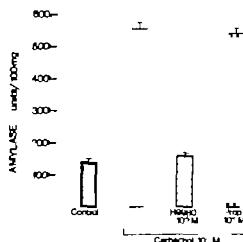


Fig 5 Amylase secretion evoked by carbachol in a concentration of 10 M The secretion of amylase is expressed as units secreted per 100 mg of parotid tissue Black column show controls Other columns show carbachol alone (open) in the presence of Hoechst 9980 (hatched) and in the presence of propranolol (dotted) Mean ±S E from 5 expts

10 ⁷ and 10^{-4} M promoted isoprenaline to release larger amounts of amylase than carbachol 10^{-4} M ($P<0.05$ $P<0.0?$ respectively) at 15 and 30 min but not after 60 min Also the pattern of isoprenaline evoked amylase secretion changed to a more rapid release in the beginning of the incubation period instead of the gradual release seen in response to isoprenaline alone and to isoprenaline in the presence of carbachol in a concentration of 10 M At these higher concentrations of carbachol isoprenaline released about 80% of its totally released amylase after 15 min and practically all of the remaining release occurred within the next 15 min There was no difference between the promoting effects of carbachol at a concentration of 10 ⁷M and that of 10 M Still higher concentrations of carbachol (10 M) regularly decreased the secretory effect of isoprenaline (10 ⁷M) The amylase secretory effect of carbachol could be completely blocked by the addition of *Hoechst 9980 at a concentration* of 10⁻ M Propranolol (10 M) did not affect the carbachol produced amylase secretion (Fig 5)

DISCUSSION

The amylase secretory effects of isoprenaline in concentrations ranging from threshold to just submaximal could all be increased in the presence of carbachol. The effect was particularly marked at low but suprathreshold concentrations of isoprenaline, at which the amylase secretory effect was increased by about 100%. The promoting effect on the isoprenaline produced amylase secretion was obtained both as an increased release of amylase into the medium and as a greater depletion of amylase from the tissue stores. This effect of carbachol was at all concentrations (10^{-8}–10^{-6} M) abolished by the atropinelike agent Hoechst 9980 (Schaumann & Lindner 1951), used instead of atropine because of the possible occurrence of atropinase in rabbit tissue (Bernheim & Bernheim 1938). It is suggested that the activation of muscarinic receptors may have in some way augmented the β-adrenoceptor mediated release of amylase. This assumption is supported by the great promoting effect that carbachol (10^{-8} M) had, which did not produce any amylase secretion on its own. At this low concentration of carbachol the augmenting effect was continuous throughout the experiment. The augmentation was obtained also at concentrations of 10^{-7} and 10^{-6} M, but mainly during the first 30 min of observation. The change in promoting effect coincides with concentrations of carbachol that are known to release K from the rat parotid gland slices in vitro (Butcher et al 1976). Since K release often is used as an indirect marker of fluid secretion in vitro (see Schramm & Selinger 1976), the change in carbachol effect may have been due t the addition of fluid secretion to the augmentation of amylase secretion. In rabbit experiments similar t the present ones rather high concentrations of carbachol and isoprenaline (both at 10^{-6} M) in combination secreted more amylase after 5 min incubation than the sum of either alone (Wojcik, Grand & Kanberg 1975). In these expts the main promoting effect of carbachol may have been due to rapid transport of amylase because of secretion of fluid. In expts using superfusion techniques high concentrations of carbachol (4×10^{-6} M) or acetylcholine (10^{-6} M) have in mouse and rat parotid tissue been shown to transiently increase the amylase secretion in response to concentrations of isoprenaline of 10^{-8}–10^{-6} M (Petersen & Ueda 1975, 1976, Harper & Brooker 1978), a result which was attributed to fluid secretion. A cholinergic augmentation of β-adrenoceptor mediated fluid secretion similar to that demonstrated in vivo for the parotid gland of the rabbit (Gjörstrup 1977) may also have contributed to the promoting effects at low agonist concentrations. However, the β-adrenoceptor mediated fluid secretion from the parotid gland in vivo is very sparce (Gjörstrup 1977) and isoprenaline is in vitro generally regarded not to cause any K -release (Batzri et al 1973). The possibility of interactions at second messenger level between the two kinds of stimuli, that would favour secretion of amylase as it occurred in these expts., has been discussed (see Berridge 1975, see Rasmussen et al 1976). It has also been speculated about the possibility of a high parasympathomimetic activity to decrease the release of amylase by lowering the intracellular levels of cAMP (Butcher et al 1976, Harper & Brooker 1978, Oron et al 1978). In the present expts an inhibition of isoprenaline evoked amylase secretion was seen at a concentration of carbachol of 10^{-} M, which alone produced an amylase secretion of the same magnitude as the highest one seen in response to isoprenaline. However, the high amylase release does not correspond to the effects in vivo of parasympathetic nerve stimulation at physiological frequencies and may indicate that the effects of such high concentrations of carbachol are of little physiological significance (Asking & Gjörstrup 1980, Gjörstrup unpublished).

In the conscious rabbit the parasympathetic and sympathetic secretory nerves of the parotid gland were found to be simultaneously activated during feeding; the main function for the sympathetic nerves was to regulate the secretion of amylase (Gjörstrup 1979*b*). Judging from a comparison between the most commonly obtained values for the amylase output during feeding expts. and those values that were obtained in acute expts. with stimulation of the sympathetic nerves, it seems as if the sympathetic activity during feeding is below 1 Hz for most of the time (Gjorstrup 1979). For low impulse traffic in the sympathetic nerves during feeding the presently described augmentation of amylase secretion may be of importance in the secretion of amylase. The regular response to a low concentration of isoprenaline (10^{-8} M) in the presence of carbachol is in accordance with the observations that very low thresholds were obtained in vivo for sympathetic stimulation (0.05 Hz) and isoprenaline injections (0.01 μg/kg) in background of

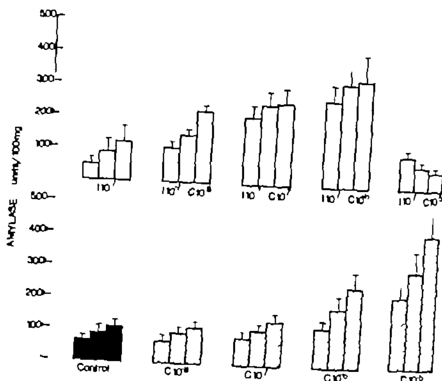


Fig 4 The hatched columns show amylase secretion in response to carbachol (C 10 –10 M) The amylase leakage from controls is shown as the black column Above is shown the net amylase secretory effects by isoprenaline (I 10 M) alone and in the presence of carbachol of different concentrations The net amylase secretory effect of isoprenaline has been obtained by subtracting the leakage in controls plus the secretory effects by carbachol in paired experiments The three columns represent 15 30 and 60 min after addition of stimulating agents The observations are the mean ±S E from 5 expts

continuous amylase secretion which was significantly larger than the leakage from controls ($P<0.05$ $P<0.02$ respectively Fig 4) Carbachol

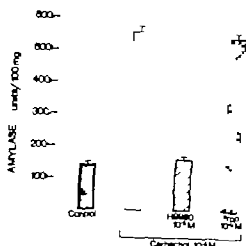


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Measurement of myometrial blood flow in rabbits by washout of Xenon 133 after atraumatic local labelling

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OTTESEN B Measurement of myometrical blood flow in rabbits by washout of Xenon-133 after atraumatic local labelling. Acta Physiol Scand 1980 109 4?1–426 Received 30 Nov 1979 ISSN 0001-6772 Institute of Medical Physiology B University of Copenhagen and Department of Clinical Chemistry Bispebjerg Hospital Copenhagen, Denmark

In vivo experiments were performed on the uterine tissue of non-pregnant estradiol treated rabbit anesthetized with sodium pentobarbitone The erage blood flow calculated from the washout of Xenon-133 after atraumatic labelling from the uterine surface was 25 4 ml·min⁻¹·(100 g) (range 7 4–66 6) and after local injection of the tracer in isotonic saline directly into the myometrium 4 4 ml·min ·(100 g) (range 8.7–45 3) During the e periment monoexponential washout curve was found for the whole w shout proces both after atraumatic labelling and when the injected olume w 5 µl No trauma of injection was observed using this injection volume The result support the applicability of a monocompartmental model for the w shout of inert gas from the myometrium Thus myometrial blood flow can be calculated from the Xenon-133 washout rate at any time interval during the washout process

Key ord Uterus, blood flow ¹³³Xenon trauma of injection

Uterine circulation has been measured in animals and man by many different method (Lewis 1969) The method include collection of the venous effluent per time unit, estimation of uterine vascular resistance at constant flow rotameter flowmeters electromagnetic flowmeters and olume plethysmography Other methods include applications of the Fick principle with N_2O or equilibration of 4-amino-antipyrine radioactive microspheres heat conductivity measurement and isotope methods using Sodium-24 tritiated water and Xenon-133 The isotope method was introduced by Kety (1949) and applied to uterine muscle with Sodium-24 by Boon et al (1963). Labelling with Xenon-133 of human skeletal muscle was introduced by Lassen et al (1964) Monck et al (1964) injected Xenon-133 dissolved in isotonic saline directly into the uterine muscle in 0 1–0 2 ml saline solution containing 50–100 µCi This technique has subsequently been used widely both in animal experiments and in human investigation Some authors report a dual multicompartment model for the non-pregnant myometrial blood flow (Forssman 1973) others monocompartmental model (Jansson 1969) In other tissues including subcutaneous adipose tissue (Le vin Nielsen 197?) and cutaneous tissue (Sejrsen 1968) monoexponential washout curves have been obtained by an atraumatic labelling technique indicating a monocompartmental model. The im of the present investigation is an attempt to measure blood flow in myometrial tissue from the washout curve of Xenon-133 following an atraumatic labelling and to compare the calculated alues with those obtained using local injection of Xenon-133 dissol ed in saline

MATERIAL AND METHODS

The experiments were performed on female rabbit of the Danish Landrace weighing 400–3 900 g, aged 140–170 days, and treated during eight days with daily intramuscular injections of 25 µg estradiol The animals were fasted for 12 h and anesthetized with sodium pentobarbitone 65 mg per kg b wt Tracheostomy wa performed and the rectal temperature was kept at 37°C by thermostatic controlled table A polyethylene catheter (Polystan, I D 0 76 mm O.D 1 22 mm) was inserted in the carotid artery for blood pressur registration and withdrawal of arterial

parasympathetic activity (Gjörstrup 1979a Asking & Gjörstrup 1980)

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In two experiments were performed on the uterine tissue of non-pregnant estradiol treated rabbit anesthetized with sodium pentobarbitone The average blood flow calculated from the washout of Xenon-133 after atraumatic labelling from the uterine surface was 25 4 ml min⁻¹(100 g)⁻¹ (range 7 4–66 6), and after local injection of the tracer in isotonic saline directly into the myometrium 4 4 ml min (100 g)⁻¹(range 8 7–45.3) During the experiment monoexponential washout curve was found for the whole washout process both after atraumatic labelling and when the injected volume was 5 μl. No trauma of injection was observed using this injection volume The result support the applicability of monocompartmental model for the washout of inert gas from the myometrium. Thus myometrial blood flow can be calculated from the Xenon-133 washout rate at any time interval during the washout process.

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MATERIAL AND METHODS

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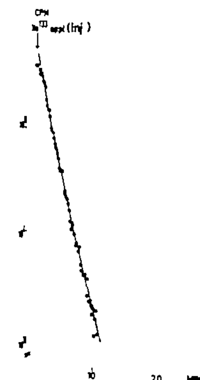


Fig. 4. The disappearance curv of Xenon-133 from the uterine horn after local intramyometrial injection of 5 μl Xenon-133. All adjacent tissue but the uterine horn w shielded off ith lead sheets.

ith NaI (Tl) crystal 32 mm in diameter and 6 mm in thickness. The detector as placed about 5 cm above the surface of the uterus. The detector was coupled to rater connection ith printer (Universal Printing Instrumenter Meditronic). A pulse-height-discriminator was used, adjusted around the 81 keV photopeak of Xenon-133. Measurement was started immediately after the

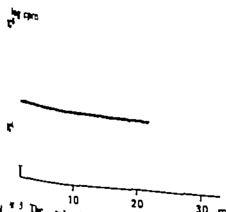


Fig. 5. The washout curve of Xenon-133 from uterine horn after atraumatic labelling, 5 min after the rabbit has been killed ith an overdose of sodium pentobarbitone.

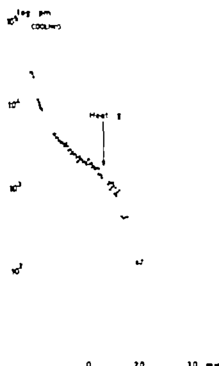


Fig. 6. The disappearance curv of Xenon-133 after atraumatic labelling during cooling followed by heating period.

isotope had been deposited and the result were printed out every 10 After subtraction of the background activity the result were plotted in semilogarithmic diagram, with the logarithm of the corrected count rate as ordinate and the time in minutes as abscissa.

Calculation

The washout of the isotope from the uterine horn was treated as monocompartmental model applying Kety (1949) classical monoexponential clearance function

$$c(t) = c( ) \exp - kt \quad c( ) \exp(-f \frac{t}{\lambda})$$

leading to

$$MBF = \frac{\ln}{T_{\frac{1}{2}}} \lambda \; 100 \text{ ml } (100 \text{g}) \quad \text{mi}$$

Where MBF is myometrial blood flow $T_{\frac{1}{2}}$ is the half time of Xenon and γ is the tissue t blood partition coefficient which was chosen as assumed to be 0.7 ml g in accordance with the figure found for skeletal muscle by Conn (1961) the tissue compositions of these tw tissues are very similar (Kao 1967 Needham & Schoenberg 1967). $k = \ln 2/T_{\frac{1}{2}}$ is the rate constant of the washout process.

Experiments

In 11 periments the myometrial blood flow was measured after atraumatic labelling. In 9 of these expts additional flow measurements were performed after labelling

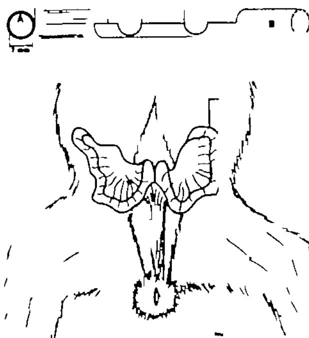


Fig 1 Upper part A spl t brass tube transverse section (A) and longitudinal section (B) Two needles transfix the mesometrium and hold the uterus in the gutter Lower part Female rabbit with the uterus exposed and one of the uterine horns placed in the split brass tube

blood samples Arterial blood pressure wa registered continuously with a transducer (Statham P 23 AA) connected to a recorder (Brush 220) Fluid replacement wa performed with isotonic saline administered by an infusion pump (Braun) connected to the right external jugular vein via a polyethylene catheter

The pH pCO_2 and pO in arterial blood were checked

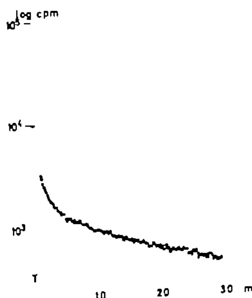


Fig 2 The disappearance curve of Xenon-133 from the uterine tissue after atraumatic labelling without any collimation with lead sheets.

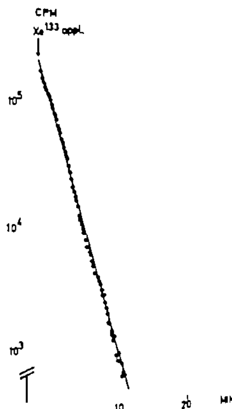


Fig 3 Same registration as in Fig All tissue b uterine horn has been covered with lead sheets.

before each blood flow measurement by a Rad me blood micro system and pH/blood gas monitor uterine horns were exposed through a midline laparot (Fig 1) Part of a horn was fixed by placing it in a brass tube provided with two needles (Fig. 1) which forated the adjoining mesometrium in vessel-free ar The inner diameter of the tube chosen was the sam the outer diameter of the uterine horn. This arrange ensured a stable geometry during the registration of Xenon-133 washout The temperature monitored b thermocouple placed near the surface of the myometri wa kept at 37°C by a heating lamp

Labell g of th t sue

The atraumatic labell ng of the uterine horn With a m yringe (1–50 µl Hamilton) 5 µl of Xenon-133 in isot saline solution (10–15 mCi/ml Kabi Sweden) was dep rted in the brass tube beneath the uterine horn form fluid coat bout 0 02 mm thick and covering an are about 2 2 cm² The local intramyometrial injection of non 133 wa performed with a needle (37G]) mounted micro yringe (1 50 µl Ham lton). The needle was int duced lmost parallel to the surface of the uterine h and advanced 1 cm 5 µl or less w injected and needle very slowly redrawn to avoid leakage The surf of the horn wa covered with a gas-tight 70 µm th Mylar membrane and moist gaze Sheets of lead (3 mm thickness) were used to shield off all t ssue act ity exc that located in the uterine horn The lead sheet did interfere with the blood supply The Xenon-133 wash sion was measured by mean of a scintillation detec

Table [illegible] Values of uterine t[illegible]sue blood flow gathered from the literature

Author	Species	Hormonal state	Technique	Myometrial blood flow (ml [illegible] min [illegible] (100 g) [illegible]) mean values
[illegible] et al (1959)	Sheep, goat	Pregnant	Nitrous oxide equilibration	22.2
[illegible] et al (1961)	Goat	Pregnant	4-Amino-antipyrine equilibration	[illegible]0.2
[illegible] et al (1964)	Human	Pregnant	Washout after injection of Xenon-133 into the myometrium	13
		Nonpregnant	Washout after injection of Xenon-133 into the portio	11
		Nonpregnant	Washout after injection of Xenon-133 into the isthmus	23
[illegible] et al (1968)	Rabbit	Pregnant	Washout after injection of Xenon-133 into the myometrium	4
[illegible] (1969)	Human	Nonpregnant	Washout after injection of Xenon-133 into the myometrium	[illegible]3
[illegible] (1969)	Rabbit	Pregnant	[illegible]Sr and [illegible]C labelled microspheres	14.5
[illegible] et al (1975)	Rabbit	Pregnant	Drop counter system	[illegible]3.6
—			Ultrasonic doppler flowmeter	27 1

(c) the tissue to blood partition coefficient for the [illegible] known

(d) the recirculation of Xenon-133 to the area [illegible] by the detector is negligible

(e) only a minor influence from disappearance of Xenon-133 by other routes than the blood (leaking between the uterine horn and the brass tube and [illegible]compartmental diffusion processes between myometrium and adjacent tissues) and

(f) only a negligible venous-arterial shunting by diffusion of Xenon-133 must take place

If these conditions are fulfilled blood flow becomes the only rate-limiting factor to disappearance of tracer from the labelled tissue and a monocompartmental model can be applied. It has previously been shown that for all practical purposes diffusion equilibrium can be obtained in the case of inert gas (Kety 1949 Sejrsen & Tønnesen 1968) Constant and homogeneous blood flow can be obtained by keeping the experimental parameters constant (temperature acid and base values blood pressure etc. The partition coefficient has previously been found to be 0 7 in striated muscle (Conn 1961) This tissue and uterine myometrium are very similar in composition (Kao 1967 Needham & Schoenberg 1967) and thus have nearly identical partition coefficients Recirculation is presumably negligible as judged from previous data (Tønnesen & Sejrsen 1970) Loss of Xenon-133 by diffusion through the narrow space between tissue and brass tube is presumably possible when atraumatic labelling is used All the washout curves observed after atraumatic labelling of the uterine horn have an almost monoexponential form during the whole washout process which is in concordance with a monocompartmental model In some expts however there was a higher disappearance rate during 1–2 min probably due to in[illegible]tial leakage of the Xenon-133 through the narrow space between the uterine horn and the brass tube Later on this had minor influence on the calculated blood flow value as concluded from smallness of the elimination rate observed after the animal had been sacrificed The washout curves after labelling by local intramyometrial injection were also monoexponential No trauma phase (Tønnesen & Sejrsen 1970) was found when the injected volume was 5 μl or less Shunting by diffusion was of no significant importance because of the relatively high blood flow values in the present experiments (Sejrsen & Tønnesen 197[illegible])

In Table 2 myometrial blood flow values have been gathered from the literature There is no report on myometrial blood flow in non-pregnant rabbits but values from pregnant rabbits and other species are of the same magnitude as the present.

The measurement of uterine blood flow by atraumatic local labelling can be applied to small animals. It is possible to use local injection of the tracer if the injected [illegible]olume is sufficiently small to avoid significant changes in tissue composition in the depot area and elevation in interstitial fl[illegible]id pressure leading t[illegible] a decrease in the perfusion of the area.

The applicab[illegible]lity [illegible]f a monocompartmental model

Table 1 *Myometrial blood flow (MBF) after atraumatic labelling and local intramy metrial injection*
The pH pCO pO and blood pressure are given for each measurement – not measured

Rabbit No	Xenon-133 application	MBF (ml (100 g min)	pH	pCO_2 (mmHg)	pO_2 (mmHg)	Blood pressure (mmHg) Systolic	Diastolic	Weight of uterus (g)
1	Atraumatic	9 1	7.5	38 3	74	110	76	6 7
	Atraumatic	51 8	7.3	35[illegible]	94	11	86	6 1
	Injection	34 7	7 4	3 [illegible]3	99	1?0	90	6 1
3	Atraumatic	18[illegible]	7 4	34 3	101	133	100	12 0
	Injection	?0	7.5	30.3	104	116	90	12.0
4	Injection	26.3	7.5	28 8	95	113	86	1[illegible]5
	Atraumatic	?9 1	7.5	[illegible]5 6	100	113	86	1 5
5	Atraumatic	18	7 4	4 8	57	106	73	7[illegible]
	Injection	15 7	7 4	38 0	68	100	73	7[illegible]
6	Injection	4 4	7.5	3 [illegible]	83	1?0	90	9 9
	Atraumatic	[illegible]5 4	–	?7 8	75	100	80	9 9
7	Atraumatic	39 8	–	4 7	79	130	90	
	Injection	?0 1	7.5	35.5	75	1?6	86	
8	Injection	8 7	7 4	36 6	83	100	73	7 6
	Atraumatic	7 4	7 4	41[illegible]	71	106	73	7 6
9	Atraumatic	1 8	7 4	40 5	78	100	80	8 5
10	Atraumatic	47 9	–	40 0	80	104	70	9 0
	Injection	45.3	–	43[illegible]	79	110	80	9 0
11	Injection	[illegible]5.3	7.4	37[illegible]	100	11	90	
	Atraumatic	66 6	–	35	74	110	80	

by direct injection of 5 μl Xenon-133 in isotonic saline into the myometrium. In expts. the temperature of the uterine tissue was varied by means of cooling (icebags) and heating (heating table and lamp) Each experiment was terminated with a period of measurement after the animal had been sacrificed with an overdose of sodium pentobarbitone

RESULTS

A representative Xenon 133 washout curve after atraumatic labelling without covering adjacent tis sue with lead sheets is shown in Fig ? This curve can be divided into ? exponentials A similar biex ponential washout curve is registrated after shielding off the uterine horn (not shown) Fig 3 shows a washout curve after shielding off all tissue but the uterine horn The latter disappearance curve of tracer can be fitted by a straight line over more than ? decades in a semilogarithmic diagram ($r = 0\ 999$ $P < 0\ 001$) During the first ? min the slope seems to be a little steeper possibly due to leakage by dif fusion of Xenon 133 out between the uterine horn and the brass tube The same monoexponential washout curve was seen after direct injection of 5 μl Xenon-133 into the myometrium (Fig 4) In prelim inary expts a larger volume (50–150 μl) of tracer solution was injected locally but this yielded biexponential curves probably due to trauma of injection

The elimination rate measured after the animal had been sacrificed (Fig 5) corresponds to less than 1.5 ml min (100 g) given as blood flow The alterations in blood flow accompanying temperature variations are shown in Fig 6 During the period of cooling the blood flow decreases gradually and the following heating period results in increasing blood flow values caused by vasodilatation Values for blood flow pH pCO_2 pO blood pressure and weight of uterus are shown in Table 1

The average flow value after atraumatic labelling was 25 4 ml min (100 g) (range 7 4–66.6) and after local myometrial injection 4 4 ml min (100 g) (range 8 7–45 3) No significant difference between the results obtained with the two methods wa found (paired ign test)

DISCUSSION

The Xenon 133 washout technique gives reliable values for blood flow only if the following conditions are fulfilled

(*a*) diffusion equilibrium between tissue and blood is obtained during the whole washout

(*b*) a constant and homogeneous distribution of blood perfusion i present

Table _ Values of uterine tissue blood flow g thered f om the literatur

Author	Species	Hormonal state	Technique	Myometrial blood flow (ml min (100 g)) mean values
...et al (1959)	Sheep, goat	Pregnant	Nitrous oxide equilibration	22._
...et al (1961)	Goat	Pregnant	4-Amino-antipyrine equilibration	20._
...et al (1964)	Human	Pregnant	Washout after injection of Xenon-133 into the myometrium	13
		Nonpregnant	Washout after injection of Xenon-133 into the portio	11
		Nonpregnant	Washout after injection of Xenon-133 into the isthmus	_3
...et al (1968)	Rabbit	Pregnant	Washout after injection of Xenon-133 into the myometrium	4
... (1969)	Human	Nonpregnant	Washout after injection of Xenon-133 into the myometrium	23
... (1969)	Rabbit	Pregnant	^{85}Sr and ^{14}C labelled microspheres	14.5
...et al (1975)	Rabbit	Pregnant	Drop counter system	23 6
			Ultrasonic doppler flow meter	71 1

(c) the tissue to blood partition coefficient for the indicator is known,

(d) the recirculation of Xenon-133 to the area seen by the detector is negligible

(e) only a minor influence from disappearance of Xenon-133 by other routes than the blood (leaking between the uterine horn and the brass tube, and intercompartmental diffusion processes between myometrium and adjacent tissues) and

(f) only a negligible venous-arterial shunting by diffusion of Xenon-133 must take place

If these conditions are fulfilled blood flow becomes the only rate-limiting factor to disappearance of tracer from the labelled tissue and monocompartmental model can be applied. It has previously been shown that for all practical purposes diffusion equilibrium can be obtained in the case of inert gas (Kety 1949 Sejrsen & Tønnesen 1968) Constant and homogeneous blood flow can be obtained by keeping the experimental parameters constant: temperature acid and base values, blood pressure etc. The partition coefficient has previously been found to be 0 7 in striated muscle (Conn 1961). This tissue and uterine myometrium are very similar in composition (Kao 1967 Needham & Schoenberg 1967) and thus ha e nearly identical partition coefficients. Recirculation is presumably negligible as judged from previous data (Tønnesen & Sejrsen 1967). Loss of Xenon-133 by diffusion through the narrow space between tissue and brass tube is presumably possible when atraumatic labelling is used. All the washout curves observed after atraumatic labelling of the uterine horn have an almost monoexponential form during the whole washout process which is in concordance with a monocompartmental model. In some expts. however there was a higher disappearance rate during 1– min probably due to initial leakage of the Xenon-133 through the narrow space between the uterine horn and the brass tube Later on this had minor influence on the calculated blood flow value as concluded from smallness of the elimination rate observed after the animal had been sacrificed The washout curves after labelling by local intramyometrial injection were also monoexponential No trauma phase (Tønnesen & Sejrsen 1970) was found when the injected volume was 5 μl or less Shunting by diffusion was of no significant importance because of the relatively high blood flow values in the present experiments (Sejrsen & Tønnesen 1972)

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The measurement of uterine blood flow by atraumatic local labelling can be applied to small animals. It is possible to use local injection of the tracer if the injected volume is sufficiently small to avoid significant changes in tissue composition in the depot area and elevation in interstitial fluid pressure leading to a decrease in the perfusion of the area.

The applicability of a monocompartmental model

for the washout process of inert gas from the myometrial tissue enables us to calculate an average myometrial blood flow at any time interval during the period of washout Thus any changes in average blood flow from one period to another can be calculated from the washout curves

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Transient vasopressin release and thirst in response to prolonged intracerebroventricular infusions of hypertonic mannitol in saline

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McKINLEY M J OLSSON K., FYHRQUIST F & LILJEKVIST E Transient vasopressin release and thirst in response to prolonged intracerebroventricular infusions of hypertonic mannitol in saline Acta Physiol Scand 1980, 109 427–431 Received 3 Dec. 1979 ISSN 0001-6772 Department of Animal Physiology Swedish University of Agricultural Sciences, Uppsala, Sweden and the Minerva Institute for Medical Research Helsinki, Finland

In the conscious goat infusions of 0.4 M mannitol in 0 15 M NaCl into the lateral cerebral ventricle (40 or 100 min 0.02 ml/min) caused slight, transient vasopressin release and temporary thirst whereas infusions of pure hypertonic (0.7 M) mannitol did not elicit thirst and inhibited the basic vasopressin release in the nonhydrated animal. In contrast, infusions of equiosmolal (0.35 M) NaCl induced persistent thirst and pronounced elevation of the plasma vasopressin concentration throughout the infusion period The cerebrospinal fluid (CSF) osmolality was raised by the same order of magnitude (~13%) after the mannitol/NaCl and the hypertonic NaCl infusions. The CSF Na concentration was elevated by >10% at 5 min after the hypertonic NaCl infusions, but it was reduced by approximately 10% at 5 min after the mannitol/NaCl infusions. There was no appreciable difference in the CSF K concentration after the infusions. The results are discussed with regard to the possible importance of CSF Na concentration as opposed to strict osmotic factors for the excitation of receptors involved in the control of water balance

Key words Intracerebroventricular mannitol NaCl, thirst, vasopressin antidiuresis, cerebrospinal fluid, goat

The question of whether cerebral receptors involved in the control of water balance primarily are sodium- or osmosensitive remains controversial (cf Andersson 1978) It has recently been suggested however that receptors of both kinds may participate in the regulation of vasopressin secretion and water intake (McKinley Denton & Weisinger 1978) This resulted from comparison of the responses in sheep to intracerebroventricular (ICV) administration of hypertonic NaCl with those to equiosmolal sucrose and fructose dissolved in artificial cerebrospinal fluid (CSF). A previously reported lack of antidiuretic and dipsogenic responses to ICV administration of pure, hypertonic sucrose solution (Olsson 1969) was confirmed whereas ICV sucrose and fructose dissolved in artificial CSF were found to cause antidiuresis and some drinking. However the antidiuretic and dipsogenic effects were considerably weaker than those obtained in response to equiosmolal NaCl These experiments in sheep supporting a dual osmoreceptor/sodium sensor mechanism involved ICV injections or brief (10 min) infusions, and the vasopressin response was assessed indirectly by studying the degree of inhibition of the water diuresis of hydrated animals It was therefore of interest to reproduce the experiments in the goat using prolonged ICV infusions combined with radioimmunoassay determination of blood plasma arginine vasopressin (AVP) In the present experiments mannitol was substituted for sucrose and fructose since mannitol appear

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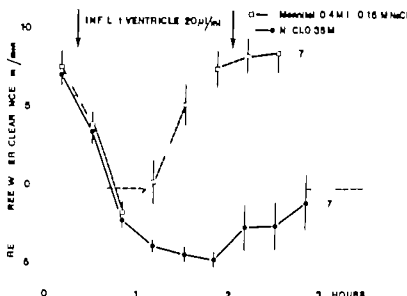


Fig 1 Marked and persistent antidiuretic response to the ICV infusions of 0 35 M NaCl (*dots*) as opposed to the weak and transient response to similar infusions of 0 4 M mannitol in 0 15 M NaCl C_{H_2O}=renal free water clearance The symbol represent mean and the bars S E

ently is equally potent as an osmotic stimulus (Szczepánska-Sadowska & Koslowski 1975)

METHODS

Animal Five female goats were used (b wts 35–55 kg) They were routinely kept in metabolism cages and were fed chopped hay three times daily Each afternoon the animals received 200 g of commercial grain mix with 6 g of NaCl added

Experimental proceeding All goats were provided with a permanent platinum-iridium cannula implanted into one of the lateral cerebral ventricles as described previously (Åkerlund Andersson & Olsson 1973) ICV infusion were made at the rate of 0 02 ml/min for 40 or 100 min periods Mannitol (0 4 M) was dissolved in 0 15 M (isotonic) NaCl and in most experiment K Mg and Ca were added to the solution in normal CSF proportions A pure 0 7 M solution of mannitol was used for ICV infusions in one series of experiment The comparative experiments involved the ICV infusion of equiosmolal (0 35 M) NaCl In hydration experiments the goats were given 100 ml/kg b wt of warm water by stomach tube into the rumen about 90 min before start of the ICV infusion In experiments involving studies of the thirst response animals displaying an urge to drink were prevented from consuming significant amounts of water during the initial part of the infusion period Thus when an animal became thirsty during an infusion a lid was put to cover the water bucket and the goat did not obtain free access to water again until the ICV infusion had proceeded for 30 min

Samples For collection of CSF a polyethylene tubing was attached to the ICV cannula with its free outlet held lower than the skull The head of the animal was slightly bent downwards with the effect that CSF dripped out of the tubing After discarding the initial three drops abo 0 3 ml of CSF was collected Blood samples were tak via a polyethylene cannula introduced into the jugular ve before the expt Samples for analyses of plasma AVP we collected in chilled tubes with 0 3 M Na-EDTA as a ticoagulant and were immediately centrifuged at +4° The plasma was stored at −20°C until radioimmunoa sayed Urine was collected in 20 min samples via a rete tion catheter introduced into the urinary bladder

Analythical method Determination of plasma AV were made according to Fyhrquist et al (1976) CSF Na and K concentrations were measured by internal sta dard flame photometry using an IL 343 flame photomete An Adv Instruments Inc osmometer was used f determination of the osmolality of the CSF

Statistic Values in both text and figures are expresse as mean and S E Student s *t*-test was employed for stati tical analyses

RESULTS

Antidiuretic effect in hydrated animals

ICV infusions (100 min) of 0 4 M mannitol in 0 15 M NaCl (N=7) were made in the pre-hydrated goat (animals) In five of the experiments K Mg an Ca were added to the infusions in normal CSF proportions The infusions rapidly reduced the re nal free water clearance (C_{H_2O}) which became nega tive within 40 min The antidiuretic effect was trans ient however Thus the water diuresis returned to pre infusion level before the end of the infusion period (Fig 1 squares) In contrast correspondin

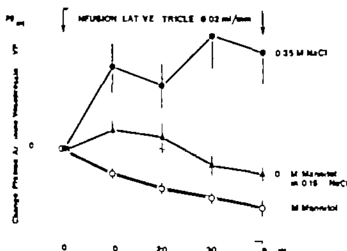


Fig 2 Persistent increase in plasma vasopressin (AVP) concentration during ICV infusion of 0.35 M NaCl. *Triangles* Slight increase of plasma AVP followed by a fall below the pre-infusion level during the ICV infusions of 0.4 M mannitol in 0.15 M NaCl *Circles* Progressive fall in plasma AVP concentration induced by the ICV infusions of 0.7 M mannitol. The pre-infusion plasma AVP concentration was 3.7±0.4 pg/ml (n 20). The symbols represent mean and the bars S.E.

ICV infusions of 0.35 M NaCl ($N=7$) induced a long-lasting inhibition of the water diuresis with C_{H_2O} remaining negative throughout the infusion period and for at least 60 min thereafter (Fig 1 dots)

Influence on plasma va opressin concentration

Plasma AVP was determined at 10 min intervals before and during 40 min ICV infusions in non-hydrated goats (4 animals) The study involved infusions of 0.4 M mannitol in 0.15 M NaCl ($N=9$) of 0.7 M mannitol ($N=4$) and of 0.35 M NaCl ($N=7$) In 4 of the mannitol/saline and 4 of the NaCl experiments K Mg^{2+} and Ca^{2+} were added to the infusions in normal CSF proportions. The mean pre-infusion plasma AVP concentrations were before infusions of mannitol/NaCl 4.4±0.6 pg/ml before infusions of mannitol/H_2O 4.1±0.9 pg/ml and before infusions of NaCl 2.5±0.5 pg/ml Since for methodological reasons, the pre-infusion concentrations varied, the induced changes from pre-infusion level are presented in Fig. 2. As evident from this figure the infusion of 0.35 M NaCl caused a marked and persistent rise in the plasma AVP (Fig. dots, increase over pre-infusion level at termination of the infusion, $P<0.01$) A transient increase in plasma AVP was observed during the mannitol/saline infusions, but here the AVP values returned to or fell below pre-infusion level within 30 min (Fig. , triangles). A progressive fall in plasma AVP was seen during infusions of 0.7 M mannitol (Fig. circles reduction from pre-infusion level at termination of infusion $P<0.05$)

Thirst

The dipsogenic response to 40 min ICV infusions of 0.4 M mannitol in 0.15 M NaCl ($N=7$) was studied in 3 goats having had free access to water until the infusions were started. The mannitol/NaCl solution contained K Mg Ca^{++} in normal CSF proportions For comparison the dipsogenic response to similar infusions of equiosmolal (0.35 M) NaCl ($N=7$) was studied in the same animals. In 3 of the mannitol/NaCl experiments no urge to drink developed during the infusion In the remaining 4 expts the animals attempted to drink 5 to 9 min after start of the infusion but were prevented from drinking until the infusion had proceeded for 30 min. In all but 1 expt the urge to drink had vanished at this stage In the exceptional expt the goat drank 200 ml of water A much stronger and persistent thirst response was obtained during the corresponding ICV infusions of equiosmolal 0.35 M NaCl. In all these expts thirst became apparent 4 to 8 min after the start of the infusion, and the goats drank

Table 1 *Changes in CSF osmolality Na and K concentrations 5 and 20 min after 40 min ICV infusions of 0 4 M mannitol in 0 15 M NaCl (N=6) or 0.35 M NaCl (N=7)*

The rate of infusion was 0 02 ml/min Mean and S E are shown

	Pre-infusion	5 min post infusion		20 min post-infusion	
		Man/NaCl	NaCl	Man/NaCl	NaCl
Osmolality mosm/kg	294±	336±7	329±5	308±3	304±2
Na mmol/l	150±1	134±	167±	143±1	15[illegible]_1
K mmol/l	8±0 0	6±0 0	2 8±0 0	[illegible]7±0 0	2 7±0 0

830±170 ml of water when obtaining free access to water after 30 min of infusion

Changes in CSF composition

Samples of CSF were taken before and after (5 and 20 min) the 40 min ICV infusions of mannitol/NaCl (N=6) and NaCl (N=7) in three animals The results of the subsequent determinations of osmolality [Na] and [K] are presented in Table 1 As shown in this table the rise in CSF osmolality observed 5 min after the infusion was not significantly different between the mannitol/NaCl and the NaCl expts As expected the [Na] was considerably elevated (>10%) 5 min after the infusions of 0 35 M NaCl However at the same stage it wa lowered by about 10% in the mannitol/NaCl expts It was still considerably below pre infusion level 15 min later A 0 2 mM reduction of CSF K was observed 5 min after the mannitol/NaCl infusions

DISCUSSION

It was originally suggested by Andersson (1971) that cerebral receptors involved in the regulation of vasopressin secretion and water intake may be sodium sensitive rather than osmosensitive (Verney 1947) and that the receptors may have a juxtaventricular location which makes them easily accessible to stimulatory and inhibitory influences exerted by the CSF composition The suggestion was based on two observations made in the goat (a) that hypertonic sucrose (in contrast to hypertonic NaCl) did not elicit water intake and apparent vasopressin release when infused into the third cerebral ventricle (Olsson 1969) and (b) that the dipsogenic and antidiuretic responses to similar infusions of angiotensin II were positively correlated to the CSF [Na] (Andersson Eriksson & Oltner 1970) Since then the experimental evidence has become considerably more substantiated that juxtaventricular sodium sensitive receptors are involved in the control of water balance ICV infusions of iso- or hypertonic saccharide solutions were found to inhibit the basic vasopressin secretion in non-hydrated normovolemic goats (Eriksson 1974 Olsson et al 1978 Fyhrquist Eriksson & Wallenius 1979) and to attenuate or extinguish the urge to drink in dehydrated animals (Olsson Larsson & Liljekvist 1976) Inhibition of vasopressin release with ICV infusion of saccharide solutions was obtained also when cations other than Na were added to the infusion in normal CSF proportions but not when the saccharides were di solved in isotonic saline This suggested that ICV administration of pure non-electrolyte solutions induced negative water balance primarily by way of a dilutional reduction of the prevailing CSF [Na] (Eriksson 1976)

The present study in goats confirms observations made in sheep (McKinley Blaine & Denton 1974 McKinley et al 1978) that CSF hyperosmolality induced by saccharides remaining in the extracellular space elicits thirst and vasopressin release if a reduction of the CSF [Na] is temporarily prevented by the simultaneous ICV administration of NaCl Therefore the present results may be regarded as additional support for the suggestion that cerebral osmoreceptors and sodium sensors interact in the control of water balance (McKinley et al 1978) Also the facts that the urge to drink vanished and the plasma vasopressin returned to or fell below pre infusion levels (Fig) during prolonged ICV infusions of mannitol/NaCl appear compatible with thi dual osmoreceptor/sodium sensor concept The pronounced decrease in CSF [Na] observed soon after the mannitol/NaCl infusion (Table 1) suggested that the disappearance of thirst and the reversal of the AVP response oc

ared concomitantly with a progressive reduction of the CSF [Na]. Most likely this fall in CSF [Na] is due to mannitol-induced osmotic movement of water from the surrounding brain tissue and the blood. However the pronounced post-infusion CSF hyperosmolality (Table 1) indicates that the addition of water to the CSF during the infusion period is not sufficient to eliminate the osmotic component of a stimulus for a tentative osmoreceptor/sodium sensor mechanism. Nevertheless the antidiuretic (Fig. 1), AVP (Fig. 2) and dipsogenic responses gradually vanished during the infusion period. Therefore, if these responses were due to stimulation of osmoreceptors in Verney's (1947) sense, it appears that the excitation mechanism of the osmoreceptors was counteracted by the effect of subnormal extracellular [Na].

A comparison between the immediate (5 min) post-infusion CSF osmolality values (Table 1) indicates that the strict osmotic stimulus component was of approximately the same magnitude during the ICV infusions of hypertonic NaCl and mannitol/NaCl. In spite of this the NaCl infusion acted as a much more efficient and persistent stimulus of thirst and AVP secretion, which also may be regarded compatible with the dual osmoreceptor/sodium sensor concept. However whether the osmotic and Na^+ stimuli act at different cells in the brain is not clear from the present experiments. The results may possibly be regarded also as support for a theory advocating a more uniform character of cerebral receptors subserving water economy. Effects obtained in response to ICV administration of inhibitors and stimulators of enzymatic transmembrane Na-transport hint at the possibility that enzymatic activity of this kind may be of crucial importance for the excitation of juxtaventricular receptors regulating vasopressin secretion and water intake (cf. Andersson 1978). It led Andersson (1978) to suggest that elevated Na concentration and cellular dehydration both may trigger a biochemical process that constitutes an essential link in the receptor excitation mechanism.

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Early effects of cervical sympathetic stimulation on cerebral ocular and cochlear blood flow

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Autoregulatory mechanisms may be expected to modify effect of vasomotor nerv stimulation in many tissues Attempt were made to reveal distinct early but transient effect of cervical sympathetic stimulation on cerebral retinal and cochlear blood flow The labelled microsphere method was used to determine regional blood flow during electrical stimulation of the cervical sympathetic chain for 15–25 and 5 min. At frequency of 6 Hz there was 5% reduction in cerebral flow at 15–25 and 7% at 5 min. In the choroid plexus the mean reduction was 22% at 15–25 but decreased to 10% after 5 min. In the cerebellum, optic nerve and retina, sympathetic timulation had no appreciable effect on the blood flow In the cochlea and iris, the blood flow reductions were 25 and 32% respectively on both occasions. In the choroid, vasoconstriction increased with time whereas in the masseter muscle there was decrease Thus in the present xperiments no indication was found of an autoregulatory escape phenomenon in the brain, the ey or the cochlea. Some escape was noted in the masseter muscle

Key ord Cerebral, ocular cochlear blood flow/cervical sympathetic stimulation/microspheres

The effect of sympathetic stimulation on the cerebral blood flow (CBF) has not been conclusively clarified Some widely different results have been published (Harper et al 1972, Heistad et al. 1977 Sercombe et al. 1975 Traystman & Rapela 1975). Using the labelled microsphere method Alm & Bill (1977) found little or no effect of stimulation at 1 min or at 15–30 min. More recently Lacombe et al. (1977) reported that ympathetic stimulation induced flow decreases of 12 29% according to structures in the brain, using the ethanol tissue sampling method in non-anaesthetized rabbits 15–30 s after stimulation started. This indicated a possibility of an early transient effect on the cerebral blood flow followed by an autoregulatory adjustment. The purpose of the present investigation was to study the effect of unilateral sympathetic stimulation on the blood flow to the brain, the eye and the cochlea in young rabbits using the labelled microsphere method (Grimm & Lindseth 1958 Wagner et al 1969 Alm & Bill 1972 t determine whether there was any difference between the effect after 15–25 s of stimulation and that after 5 min

MATERIAL AND METHODS

Ten young rabbits of both sexes weighing between 1 4-3 0 kg were used The animal were anesthetized with pentobarbital sodium 60 mg/kg tracheotomized and ventilated by Palmer pump They were placed on servocontrolled heating pad and had temperature around 38°C Both femoral arteries were cannulated, one for continuous blood pressure measurements and the other for blood sampling Both sympathetic cervical trunks and both vagal nerves were identified and cut above the thoracic aperture The sympathetic nerve on one side was carefully prepared, connected to bipolar silver electrode isolated from the surroundings to avoid timulation of other nerves. The stimulation frequency was 6 Hz, intensity 7 V and duration 2 ms The effect was judged by the pupillary response. The frequency 6 Hz is supposed to exist physiologically under moderate to intense stres conditions (Mellander & Johansson 1968) and produces almost maximal pupil dilatation. After thoracotomy and control of the acid-base balance the stimulation was started. After 15 of stimulation the first dose of microspheres was

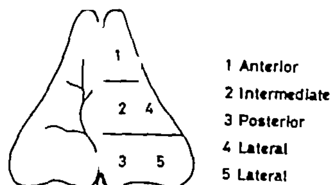


Fig 1 Dissection of the brain into the different parts

injected into the left ventricle of the heart and the blood sampling was started (six 10-second samples) from free flow of the femoral artery at the same time The injection time was about 10 s and the sampling time always exactly 60 s The stimulation wa continued and after another 5 min the second injection of microspheres was made using differently labelled microspheres Blood wa sampled for 60 s and the animal was put to death by intracardial injection of KCl

The brain the eyes the membranous parts of the cochleas (Angelborg et al 1977) and samples of the masseter muscles were dissected out The blood samples were weighed and the radioacti ity was determined with a gammaspectrometer The blood flow was calculated using the formula

$$\frac{f_t}{n_t} = \frac{f_{ref}}{n_{ref}}$$

where f_t=blood flow in the tissue g m n f_{ref}=the reference blood flow g min n_t=radioactivity of the tissue and n_{ref}=radioacti ity of the reference blood sample The regional blood flow on the stimulated and non-stimulated sides were compared Each hemisphere of the brain was dissected into 5 parts representing anterior posterior intermediate and two lateral regions (Fig 1) The ciliary body was separated from the iri About 7 mm of the distal part of the optic nerve wa taken a one sample

Microsphere

The microspheres used were 9±0 9 μm 8 7±0 6 μm and 15±1 0 μm in diameter and labelled with ¹Ce and ⁸⁵Sr (3 M Co St Paul Minnesota) In 7 expt both measurements were made with small spheres in number of about 17×10^6 in each injection In 3 expts the first injection consisted of spheres 15 μm in diameter in a number of 17×10^6 spheres and the second injection of 17×10^6 spheres with 8 7 4×10^6 and the second injection of 17×10^6 spheres with 8 7 μm in diameter Large amounts of spheres were used to get a sufficient number into the cochlea (Buckberg et al 1971) The experiments with two sizes of spheres were performed to check whether the results were dependent on the size of the spheres used The spheres were suspended in saline and 10% Dextran aggregates were di rupted using a Potterhjelm homogenisat r Each injection was 1.5–2 ml

RESULTS

All the animals tolerated both the anesthesia and the surgical manipulation well The mean arterial blood pres ure and values for the acid-base balance are presented in Table 1 In the 3 animal where 15 μm spheres were used in the fir t injection the blood pressure was unstable during the first 10 s but the volume of the blood samples collected during these 10 s was similar to the subsequent samples During the second injection the blood pressure was stable Mydriasis was near maximal within 15 s of sympathetic stimulation

Values for the mean blood flow in the different tissues are presented in Tables 2 and 3

Paired comparisons between the blood flow on the stimulated and non stimulated side were performed in each experiment and the differences were expressed as percentages of the flow of the non-stimulated side During the first measurement the mean difference ±S E between the two hemispheres of the brain was 5 0±2 1% This difference was significant (P<0 01) using Student s t-test for paired comparisons During the second measurement the difference between the stimulated and the control side was 7 0±2 5% (P<0 01) During both injections the blood flow was about 15% higher in the posterior part of the brain than in the anterior part (Fig 1) (P<0 05 using the t-test for paired comparisons)

In the anterior part no significant difference in blood flow was seen between the stimulated and the

Table 1 *Acid-base values before and mean arterial blood pressure during the blood flow measurements*

Exp no	MAP cm H_2O Inj I	Inj II	pH	P (kPa)
1	145	170	7 4	5 31
	95	85	7 37	6 19
3	165	130	7 59	4 34
4	130	130	7 57	5 65
5	110	105	7 47	4 79
6	90	115	7 31	4 98
7	145	135	7 43	4 33
8	130 un table	170	7 47	4.31
9	130 un table	105	7.34	3 45
10	170 unstable	100	7 45	4 0[illegible]

Table Blood flow in g min g in different tissue during unilateral cervical sympathetic stimulation (mean S E 10)

Sam tissue	15–25 Stim side	Contr side	5 min Stim. side	Contr side
Cerebrum	0.39±0 03	0 41±0 03	0.39±0 03	0 43±0 03
Anterior part	0 38+0 03	0 47±0 03	0 43±0 03	0 44+0 03
Posterior part	0 43+0 03	0 47±0 03	0 45±0 03	0.50_0.03
Cerebellum	0 45±0 03	0.42±0 03	0 47_0 03	0.48±0 03
Choroid plexus	[illegible]±0 58	2.78±0 68	1.86_0.53	2.07_0.57
Optic nerve	0 52+0 08	0.53±0.10	0 51_0 09	0.55+0 13

non-stimulated side, and there was no difference between the first and second measurement In the posterior part there was a difference of approximately 10% between the stimulated and non-stimulated side which was significant at the 5% level at both occasions

No significant effect of sympathetic stimulation on the blood flow could be seen in the cerebellum the optic nerve or the retina. In the choroid plexus an initial difference of 22 0±6.6% ($P<0\ 01$) between stimulated and non-stimulated sides was noticed using paired comparisons After 5 min there was no significant difference

In the choroid, blood flow was initially reduced on the stimulated side by 26.0±1_0% and by 48+9.5% 5 min later The corresponding figures for the iris were 33 0±21 0% and 41 0±18 0% The cochlear blood flow was reduced by 33 0±8 0% on the stimulated side at the first injection. This difference was significant ($P<0\ 05$) After 5 min the difference was 24 0±6 0% ($P<0\ 05$) There was a good agreement between the flow values calculated from data for 8.7 μm spheres and calculations using data for 15 μm spheres

The blood flow values for the masseter muscle are presented in Table 4 In 8 expts of 10 the blood flow on the stimulated side was almost stopped during the first measurement and then increased

DISCUSSION

Histological studies by Nielsen & Owman (1967) on cats have shown that the anterior cerebral artery and cerebellum receive adrenergic innervation from the superior cervical ganglion both on the ipsilateral side and to some extent from the contralateral side But Helstad et al (1978) stated that the functional importance of innervation to the contralateral hemisphere is not great. Lacombe et al (1977) demonstrated that CBF in non-stimulated rabbits did not differ from CBF on the non-stimulated side in rabbits exposed to unilateral stimulation However according to their study there might be some nervous cross-over in the vessels supplying the optic nerve In the inner ear of the cat part of the vascular innervation seems to originate in the contralateral stellate ganglion (Spoendlin 1969) but Densert (1974) showed that the sympathetic innervation of the rabbit cochlea derives only from the ipsilateral superior cervical ganglion. As these latter functional studies showed no larger cross-over of the sympathetic nerves, rabbits were used in this study

For blood flow measurements in small organs, such as the cochlea, it is necessary to use large amounts of spheres This is possible when using small spheres, but if they are too small the spheres may pass through the tissue Recent studies indi-

Table 3 Blood flow in whole tiss g m during unilateral sympathetic stimulation (mean ± S.E. n 10)

Tissue type	15–25 Stim side	Contr side	5 min Stim. side	Contr side
Retina	5 66 1 76	6 32±1 14	3 96±1 14	5.50±0 93
Iris	33 48±8.10	49.68±9.90	21.89±5 73	31 98±9.5
Choroid	660.70±88	848 73±85	334 11±54	639.20±85
Cochlea	2.5 ±0 38	3.33±0 41	2.9 ±0 35	3 81+0 44

Table 4 *Blood flow in g min⁻¹ g⁻¹ in the masseter muscle during unilateral cervical sympathetic stimulation*

Measurements after 15–25 s (inj I) and 5 min (inj II)

Exp no	Stimulated side Inj I	Stimulated side Inj II	Control side Inj I	Control side Inj II
Small spheres both injections				
1	0 0081	0 79	0.26	0 15
2	0 0011	0 016	0.26	0 1
3	0 009?	0 10	1 04	0 48
4	0 11	0 086	0..5	0 13
5	0 0002	0 0039	0 49	0.30
6	0 0009	0 023	0 .3	0 29
7	0 0005	0 0076	1 44	0.36
Large spheres first injection				
8	0 15	0 078	0 60	0 11
9	0 001	0 027	1 08	0.39
10	0 004	0 009	0 39	0 14

cate that 8–10 μm spheres tend to pass through the iris and ciliary body (Stjernschantz Alm & Bill 1976) and that 8 2±0 8 μm spheres tend to pass through the brain (Hultcrantz 1979) In the present study no clear tendency of passage was seen with 9 0 or 8 7 μm spheres in any of the organs investigated

The differences in mean arterial blood pressure in the animals were probably at least partly due to different ages and maturation but it is well known that cerebral blood flow is efficiently autoregulated within a wide range of blood pressures In the eye of the rabbit the retina is well autoregulated but in other parts autoregulation is poor The cochlea also has a rather constant flow at normal blood pressures (Hultcrantz et al 1977) Alm & Bill (1973) have shown that unilateral electrical stimulation of the cervical sympathetic chain has little or no effect on cerebral and retinal blood flow Under the same conditions Hultcrantz & Angelborg (1977) measured a 25% reduction in cochlear blood flow The microsphere method was used on cats in both these studies Lacombe et al (1977) using the ethanol technique and the local thermoclearance technique in unanesthetized rabbits reported reductions in flow of 12–29% during sympathetic stimulation the effect varying from one place to another Lassen (1974) reviewed the literature on sympathetic effects on cerebral vessels and concluded that stimulation probably gives a slight vasoconstriction and a flow reduction of about 10% in normotension Ou[r] results agree with this view In hypertension a mor[e] pronounced vasoconstriction is observed durin[g] sympathetic stimulation (Bill & Linder 1976)

When microspheres are used the time investi[-] gated equals the injection time plus a few second[s] since the spheres disperse almost immediately ou[t] into the tissues The microsphere technique used i[n] the present expts thus enabled registration of th[e] transient flow change occurring 15–25 s after stimu[-] lation started This instant of time interval seeme[d] the most interesting since prolonged stimulation o[f] the sympathetics might result in a reduction of th[e] response an "autoregulatory escape" In the inte[s-] tine the maximal constrictor effects appears at 20[-] 25 s (Dresel et al 1966) whereafter it decreases I[n] the present study a possible autoregulatory escap[e] of this kind could only be seen in the massete[r] muscle (see Table 4) This tissue has a very ric[h] sympathetic innervation causing vasoconstrictio[n] with an extreme reduction in blood flow whe[n] stimulated (Table 4) as seen also in previou[s] studies (Alm & Bill 1973 Hultcrantz & Angelbor[g] 1978)

It is somewhat surprising that the effect of sym[-] pathetic stimulation seemed to be more marked i[n] the posterior part of the brain than in the anteri[or] part (Table ?) The anterior part has been reporte[d] to have a richer adrenergic innervation than th[e] posterior part (Owman et al 1974) The lack o[f] effect of sympathetic stimulation on the blood flo[w] in the retina and the optic nerve shows good agre[e-] ment with previous reports (Alm & Bill 1973) Thu[s] in normotension it would not appear probable th[at] the sympathetics play a role in the regulation of th[e] blood flow in the retina and the optic nerve A[n] important vasoconstrictive effect and protectio[n] against overperfusion has been observed in acu[te] arterial hypertension (Bill Linder & Linder 1977)

The role of the sympathetic nerves in the contr[ol] of cochlear blood flow is not known The observa[-] tion of Borg (1977) that noise tends to cause vas[o-] constriction in the tail of the rat might also apply t[o] the cochlear blood circulation However recent re[-] sults by Hultcrantz (1979) showed no vasoconstri[c-] tive effect of noise on the cochlear blood flow Further studies are planned to study whether th[e] sympathetic nervous system plays a role in protec[-] tion against overperfusion during acute hyperten[-] sion as in the brain

Thus no indication was found for an auto[-]

regulatory escape phenomenon in the brain the eye or the cochlea of anaesthetized rabbits.

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Transfer properties of the slowly adapting stretch receptor of the crayfish abdomen

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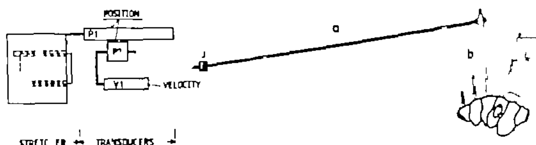
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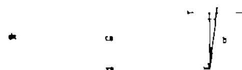
The slowly adapting stretch receptor in the abdomen of freshwater crayfish (Astacus fluviatilis) was investigated to determine its properties under dynamic conditions. An in situ preparation was used; the necessary dissection did not involve the receptor organ or its immediate surroundings. Sinusoidal variations in the angle of flexion in the joint to which the receptor organ was connected were generated by a feed-back controlled stretcher. Nerve spikes recorded from the axon of the receptor neurone and information about angle of flexion in the joint obtained by position transducers, were fed into a computer. Fourier transforms were performed on both input and output data to determine the amplitude of the 0. and 1. harmonic together with the phase of the 1. harmonic. The receptor organ was investigated for linearity up to 1.5 degrees input amplitude and proved to be surprisingly linear within this range. In addition, the transfer function of the receptor organ was determined by stimulating it with small-amplitude sinusoidals with different frequencies. With a steady flexion of 35–40° in the joint, the gain of the receptor organ increased 5–6 times when the modulation frequency of the input signal was increased from 0.1 to 5 cycles/s. A maximum in gain was constantly found at about 5 cycles/s, with a rapid fall towards 0 when the modulation frequency was increased further. A change in phase lead from positive (leading output) to negative with change in sign about 1 cycle/s was also found. These results resemble the results found by investigators of isolated preparations. A "hold" property is probably a part of the overall property of the receptor organ together with an element of Maxwell type. An element of the form $h(s)=ks^{a}$ with $a\approx0.45$ is also part of the transfer function of the receptor organ, although the physiological parallel to this element is uncertain.

First described by Alexandrowicz (1951), the stretch receptor organ of the crustacean abdomen has been the subject of many investigations, anatomical (for references see Alexandrowicz 1967) as well as physiological (for references see Terzuolo & Knox 1971). In crustaceans each abdominal segment is equipped with two pairs of receptors situated dorsally, symmetrically near the midline. Each receptor pair consists of one rapidly adapting and one slowly adapting receptor. Only the slowly adapting receptor has been studied in the present investigation.

Any receptor organ may be regarded as a system which transforms one kind of information (input) into another form (output). If the system is linear, it can be represented by a transfer function, which is a particularly useful way of describing the transfer properties. The transfer function may be found by the study of the response of the system to certain input signals. Sinusoidals are widely used signals in such investigations, as well as white noise signals. The response of a linear system to a sinusoidal input will be sinusoidal, while the response on nonlinear systems is normally not a sinusoidal.

The transfer properties of the isolated receptor organ of the crayfish abdomen have been investigated earlier, both under static and dynamic conditions (Terzuolo & Washizu 1962, Brown & Stein 1966). These authors found a linear relationship between the length of the receptor muscle and the firing frequency of the slowly adapting receptor under static stretches. However, Njå & Walløe (1973) investigated the receptor in situ under static stretches and found a power-function relationship with exponent equal to 2 between input and output. Whether or not the dissection and preparation





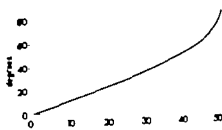


Fig 1 (A) Experimental setup To the right the crayfish abdomen showing the site of dissection of the nerve from the stretch receptor To the left feedback controlled stretcher and transducers P1 position transducer measuring position of stretcher P2 position transducer (differential transducer) measuring position of the coil of the stretcher V1 velocity transducer P2 and V1 gives feedback to the controller J marks ball joint coupling between stretcher and preparation. The position is measured by P1 and P2
(B) Geometrical representation of the setup Origin corresponds to point J (see above) a, b co and xo are measured on the setup dx difference from zero position of point J measured by P1 and P2, α corresponding angle of flexion in the joint
(C) α as a function of dx the actual values measured on a setup were a=150 4 mm b=49 9 mm co=150.8 mm, and xo=144 8 mm A linear relation between dx and α in the physiological range (0–42°) is seen

methods used to obtain the isolated preparations explain the differences between these earlier investigations is uncertain The non-uniformity of the receptor muscle bundle found both anatomically (Bodian & Bergman 1962) and physiologically (Wendler 1963) indicates however that any manipulation of the receptor muscle might well interfere with the transfer properties of the receptor organ

We therefore decided to re-examine the transfer properties of the receptor organ under dynamic conditions using an in situ preparation

Some of the results have been presented to a meeting of The Scandinavian Physiological Society (Sydnes 1979)

METHODS

Dissection and mounting of the preparation

The experiments were performed on freshwater crayfish Astacus fluviatilis Removal of the thorax left an isolated abdomen which could be kept alive for hours. During the dissection the abdomen was kept in cold saline (5–10°C) containing (mmol/l) NaCl 205 KCl 5 4 $CaCl_2$ 13.5 $MgCl_2$ 6 Tris 10 titrated to pH 7.–7 4 with maleic acid. A hole was bored in each tergite in the dorsal midline and glass rods screwed into the holes Dental cement was used to fasten the glass rods and fix all joints except the third in zero position as shown in Fig 1 A hole was then drilled in the third tergite on the right side of the abdomen (see Figs 1 and) and the underlying connective tissue membranes were removed Usually the nerve containing the afferent axon from the receptor could easily be found embedded in the loose connective tissue covering the large flexor muscle bundle This nerve was dissected free over the greatest possible length and cut centrally After dissection the preparation was moved to a bath containing oxygenated saline with thermostatically controlled temperature of 11°C and the glass rod of the fourth tergite was coupled to a stretcher The central end of the cut nerve was drawn into a suction electrode and the signal fed to an amplifier

The resulting experimental setup is shown in Fig 1A. The stretcher was feedback controlled by signals from one position and one velocity transducer The reference signal was generated by a Wavetek 184 signal generator Information about the position in the joint was obtained from two position transducers one of which measured large movement the other small fluctuations in joint angle The latter was also part of the feed-back loop controlling

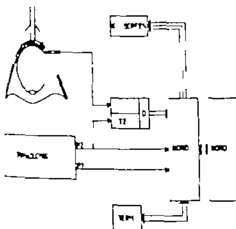


Fig. 2. Coupling between setup and computers (NORD-1 and NORD-10). Transducer symbols as in Fig. 1 A. T1 and T2 one-level trigger parts of lab-box. D' digital part of lab-box.

the stretcher. Both transducers were calibrated and found to behave linearly. Using calibration data, every change in angle of flexion in the third abdominal segment could be obtained from the geometry of the system, as shown in Fig. 1 A and 1 B. The curve in Fig. 1 C shows the angle of flexion as function of deviation in point J (Fig. 1 A) from zero position with parameters from an actual preparation. Calculations showed that this curve changed very little as long as the changes of the parameters were reasonable and that the function was very nearly linear within the physiological range of the angle of flexion (0–42°).

Stimulation of the receptor

The experiments were performed in the following manner: (1) The stretcher was manually moved to a preset position, resulting in a steady flexion in the joint of 35–40°. This steady stretch was measured by transducer P1 (Fig. 1 A). (2) After 15 s of adaptation, a sinusoidal voltage starting at zero was sent to the reference input of the feedback controller of the stretcher. This resulted in a sinusoidal output from transducer P2 (Fig. 1 A), and hence a sinusoidal variation in angle of flexion around the steady flexion in the joint. The traces and *b* in Fig. 3 A show the voltage from P2 together with the resulting spike train with its periodic variations.

Computer hardware

The preparation was coupled to the computer via one digital and two analog lines as shown in Fig. 2. The analog lines were read in a conventional way by a NORD-1 computer, and carried signals from the position transducers, thus enabling calculation of the angle of flexion in the joint at any wanted moment. The digital line carried information from a level-discriminator giving "trigger signals" whenever its preset level was passed in one direction. Our level discriminator had two separate inputs, one of which received the spike train from the nerve. The signal from

transducer P2 was sent to the other input, thus generating a cycle-marking signal.

A FORTRAN written program was running in NORD-1 during the experiment, enabling on-line data processing and presentation as well as storage. On-line data were presented on an TV-screen, and the action of the program controlled through a teletype (see Fig. 2).

Each triggering event arriving at the level-discriminated circuit was timed by a clock-procedure. In our program, it was programmed to count millisecond intervals. This proved to give time resolution good enough for our purposes, since the firing frequency only seldom was higher than 20 imp/s.

Data processing

The data processing consisted of different modules which could be activated by commands from the terminal. Processing of data for presentation of instantaneous frequency was, however, done while nerve spikes were collected by the computer. The presentation of these data is shown in trace of Fig. 3 A, where the instantaneous frequency corresponding to each interspike interval of trace *b* is plotted, showing nearly sinusoidal variation in the instantaneous frequency. The aim of the further data processing was to determine relevant parameters for this cyclic variation in the instantaneous frequency together with parameters characterizing the input sinusoidal.

To obtain good estimate for the parameters of the cyclic variation of the output, it was necessary, especially at high modulation frequencies, to read the nerve impulse train over many input cycles. This was done by presetting the total reading time, normally to 20 s. This would result in a nearly constant number of registered spikes, distributed over an increasing number of stimulation cycles when cycle time decreased. Trace in Fig. 3 B shows the instantaneous frequency plotted one cycle upon the other by resetting of the plotting procedure each time the cycle mark arrived.

Each cycle was divided into a number of bins, in these experiments 16, to make possible later analysis of our data by Fast Fourier Transform. Each spike was referred to one bin, and the mean of the interspike intervals calculated for each bin. Inversion of the value referred to a bin gave a good estimate for the firing frequency corresponding to the midpoint of it. The set of values obtained in this way was then used as input to the fitting procedure.

At the end of the spike reading, the cycle length was known to the computer. This was then used for reading the input signals from transducer P1 and P2, shown in Fig. 1. 16 analog values were read from the cyclic signal of P2 together with the corresponding times, followed by the reading of P1. From these data it was possible to calculate the angle of flexion at 16 moments in one cycle. The Fourier transform was used for finding the parameters of the two cyclic signals, giving information about the 0. and 1. harmonic together with the phase of the 1. harmonic. The results obtained by this procedure are shown in trace *d* in Fig. 3 B, where the continuous line shows the best sinusoidal fit to the 16 bin values, indicated by crosses. Trace shows difference in phase found between input and output. Although it would be easy from a mathematical point of view, no higher harmonics were obtained

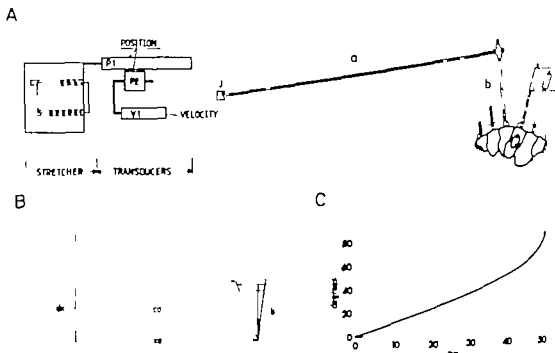


Fig 1 (A) Experimental setup. To the right the crayfish abdomen showing the site of dissection of the nerve from t stretch receptor To the left feedback controlled stretcher and transducers P1 position transducer measuring positi of stretcher P2 position transducer (differential transducer) measuring position of the coil of the stretcher V1 veloc transducer P2 and V1 gives feedback to the controller J marks ball joint coupling between stretcher and preparatio The position is measured by P1 and P2.
(B) Geometrical representation of the setup Origin corresponds to point J (see above) a, b co and xo are measured the setup dx difference from zero position of point J measured by P1 and P2 α corresponding angle of flexion in t joint.
(C) α as a function of dx the actual values measured on a setup were a=150 4 mm b=49 9 mm co=150 8 mm a xo=144 8 mm A linear relation between dx and α in the physiological range (0–42°) is seen

methods used to obtain the isolated preparations explain the differences between these earlier investigations is uncertain The non uniformity of the receptor muscle bundle found both anatomically (Bodian & Bergman 1962) and physiologically (Wendler 1963) indicates however that any manipulation of the receptor muscle might well interfere with the transfer properties of the receptor organ

We therefore decided to re-examine the transfer properties of the receptor organ under dynamic conditions using an in situ preparation

Some of the results have been presented to a meeting of The Scandinavian Physiological Society (Sydnes 1979)

METHODS

Dissection and mounting of the preparation

The experiments were performed on freshwater crayfish Astacus fluviatilis Removal of the thorax left an isolated abdomen which could be kept alive for hours. During the dissection the abdomen was kept in cold saline (5–10°C) containing (mmol/l): NaCl 205 KCl 5 4 CaCl 13.5 MgC 2 6 Tri 10 titrated to pH 7 7 4 with maleic acid. A ho was bored in each tergite in the dorsal midline and gla rods screwed into the holes Dental cement was used t fasten the glass rods and fix all joints except the third zero position as shown in Fig 1 A hole was then drilled i the third tergite on the right side of the abdomen (see Fig 1 and) and the underlying connective tissue membran were removed Usually the nerve containing the affere axon from the receptor could easily be found embedded the loose connective tissue covering the large flexor mu cle bundle This nerve was dissected free over the greate possible length and cut centrally After dissection th preparation was moved to a bath containing oxygenate saline with a thermostatically controlled temperature 11°C and the glass rod of the fourth tergite was coupled t a stretcher The central end of the cut nerve was draw into a suction electrode and the signal fed to an amplifier

The resulting experimental setup is shown in Fig. 1A The stretcher was feedback controlled by signals from on position and one velocity transducer The reference sign was generated by a Wavetek 184 signal generator Info mation about the position in the joint was obtained fro two position transducers one of which measured larg movements the other small fluctuations in joint angl The latter was also part of the feed-back loop controlle

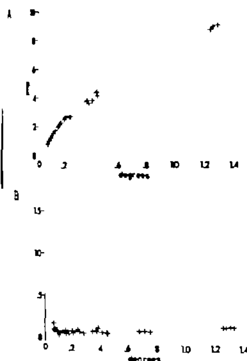


Fig. 4. Linearity of the receptor response. (A) Output amplitude as function of input amplitude. Modulation frequency 1 cycle per second, steady stretch about 38° angle of knee. (B) Noise-to-signal ratio in the output as function of input amplitude. The relationship is calculated as absolute difference per point divided by amplitude of first harmonic. Same experiment as in A.

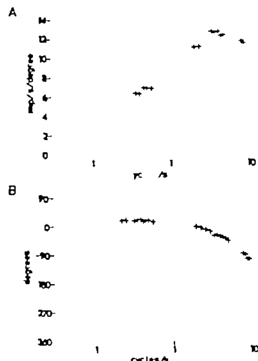


Fig. 5. Transfer properties of the receptor organ. (A) Gain as function of the modulation frequency in semi-logarithmic plot with linear vertical axis. Steady stretch = 38°, input amplitude 0.2°. (B) Phase difference between input and output as function of the modulation frequency. Positive phase difference correspond to leading output.

ment, plotted in two semi-logarithmic diagrams. The upper part of the figure (A) shows the gain as a function of modulation frequency, showing a four fold increase in amplification over the range of 1 to 5 cycles per second. This large increase was a constant finding. The maximum was constantly found around 5 cycles per second. The experiments were always performed with modulations around a steady stretch of 35–40°, giving almost the same mean firing in every experiment. Therefore we have not made any corrections or transformations of the frequency-axis as other investigators have done (Borsellino et al. 1965). One reason for always using a large steady stretch was the relatively low firing rate of the receptor after the initial adaptation. Lower steady stretch would result in an unacceptably low rate of firing, and silent periods during the cycle, introducing greater uncertainty in the estimates of the parameters.

As one would expect taking phase locking phenomena into account, the gain falls rapidly towards zero when modulation frequency is increased towards the mean firing-frequency of the receptor. This behaviour, also found by other investigators, is usually interpreted as an effect of the "holding" properties of the coding process (Borsellino et al. 1965, Terzuolo & Knox 1971). To obtain information on transfer properties near this point, it is necessary to prevent phase locking. This may be done either by applying very small amplitude inputs to the receptor, or by adding noise to the input signal.

The lower part of Fig. 5 shows the phase difference as a function of the modulation frequency. The phase difference is defined as positive when the output leads to the input (as is shown in Fig. 3 A). Fig. 3 B shows a registration with negative phase difference (trace f). Regarded isolated, the positive phase difference might be interpreted as an effect of velocity-sensitivity in the receptor. A constant el-

during the on-line computations Neither were attempts made to fit sinusoidals directly to the instantaneous frequency data.

RESULTS

Linear behaviour of the receptor

We decided first to find how linearly the receptor behaves under dynamic conditions This was done by introducing sinusoidals with constant frequency but with different amplitude to the receptor

Fig 4 A shows the results from one such experiment with modulation frequency 1 cycles/s The steady flexion in the joint was in this case about 38° and the mean firing frequency after adaptation for 15 s slightly above 10 impulses per second A reading time of 20 s gave a total number of registered nerve spikes of about 210 or about 13 spikes per bin using 16 bins in the analysis of the spike train The upper limit of the input amplitude (in this experiment about 1.3°) was partly determined by the movements of the nerve at the site of recording Greater amplitudes gave instability in the recording and problems with the spike discrimination At low values of the input amplitude the output became increasingly noise-dominated

We also investigated alternative measures of linearity Noise to-signal ratios based on both quadratic and absolute difference were examined together with higher harmonics The noise to-signal relation expressed as (absolute difference per point)/(amplitude of first harmonic) is plotted as function of input amplitude in Fig 4B The high value of this parameter for small input amplitudes indicates noise domination The slight increase in noise fraction when input amplitude was increased was also found in the other measures When the lowest amplitude values were excluded the higher harmonics (2–7) all seemed to be linear functions of input amplitude Expressed by means of amplitude of the first harmonic (A 1) a fairly constant relation seemed to be A2=A1/5 and for n between 3 and 7 An=A1/20

The results indicate an approximately linear behaviour of the receptor under dynamic conditions This was a common finding in our experiments also at other modulation frequencies We also constantly found a slight decrease from strict proportionality when input amplitude was increased as illustrated in Fig 4A indicating a fall in the gain of the receptor for large input amplitudes

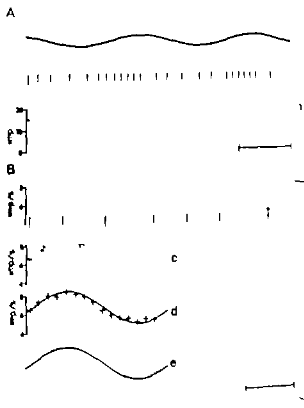


Fig 3 (A) Registration from receptor with mean firing frequency of about 10 imp/s. Duration of modulation cycle 1 s. Trace *a* Output from position transducer P (see Fig 1) as a function of time Amplitude approximately 0 8° Trace *b* Corresponding spike train from the receptor showing periodic variation in interspike intervals. Trace *c* Corresponding intantaneous spike frequency
(B) Data obtained from a receptor with mean firing frequency of about 7 imp/s Duration of modulation cycle 0 5 s Input amplitude 0 15° Trace *a* Instantaneous spike frequencies corresponding to spike train in trace *b* Trace *b* Reconstructed spike train Trace *c* Instantaneous spike frequencies of all interspike interval in the experiment plotted with resetting of the abscissa. Trace *d* Crosses show the mean interspike interval in each of 16 bins. Fully drawn line shows the best sinusoidal fit to the experimental points Trace *e* Best fitted sinusoidal from trace (fully drawn line) and output from position transducer (P dotted line) plotted with arbitrary vertical scale to show the phase difference between the two In this case the input to the receptor leads to the output from it, a situation referred to a negative phase difference here −9 6°

Transfer properties

The transfer properties of the receptor organ were studied by using small sinusoidals with different frequencies as input to the receptor In about 15 expts we tried to keep the amplitude approximately equal to 0 1–0 2° corresponding to an amplitude in the receptor muscle length of 5–10 μm (Njå & Walløe 1973) Fig 5 shows the result of one such experi-

scribed elements of the form $h(s)=1/(s^2+2\gamma ps+p^2)$ in the system, concluding from finding a flat resonance in the logarithmic plot that the damping factor γ is fairly large. The suggested value of both p and γ is 0.7 in their paper. This transfer element corresponds to a viscoelastic Maxwell-element which might be a property of the receptor muscle. The points to the extreme right in Fig. 6 A and B do agree with the expected effect of a Maxwell-element. The location of the maximum gain in Fig. 6 A corresponds, with its maximum slightly below 10 cycles per second, to the findings of previous investigators. Both gain and phase data, however, suggest a lower damping factor than that found by Borsellino and co-workers (1965) in the isolated receptor. This difference may be explained as by damage to the receptor muscle in the isolated preparation.

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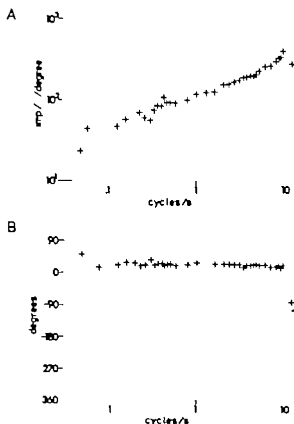


Fig 6 Transfer properties after subtraction of zero order hold element with sampling time T = 1/13.3 s. (A) Gain data from Fig 5 A after division with the expected amplitude of a hold element. A double logarithmic plot shows nearly linear relation in this system. (B) Phase data from Fig 5 B after subtraction of expected effect of hold element

ocity sensitivity cannot however explain the fall in phase difference which is observed when the modulation frequency is increased In our experiments the phase difference changed sign from positive to negative at slightly above 1 cycle per second

Fig 6 shows gain and phase difference respectively when the effect of the zero order hold element has been subtracted The fairly linear increase of the gain function is obvious as in the horizontal course of the phase difference

Finally one factor which influences the phase lag ought to be mentioned namely the conduction time of the nerve spike from site of generation to site of recording From previous investigations on the same preparation we have estimated the conduction time to about 5 ms This would give a small negative phase difference increasing with increasing modulation frequency The phase effect of this would be $\pi/20 = 9°$ when modulation frequency is 10 cycles/s Hence this effect may be disregarded

DISCUSSION

The results show that the slowly adapting stretc receptor behaves quite linearly in the amplitu range from 0 to 1 5° when it is modulated around steady flexion in the interval 30 to 4?° For small steady flexion the linearity property is difficult t investigate by the present methods.

The other main result from the investigation i that the slope of the proportionality line betwee input amplitude and output amplitude (as shown i Fig 4 A) is about 6 times steeper for modulatio frequencies near 5 cycles per second than for mo ulation frequencies near 0 1 cycles per second.

Most investigators agree that properties resemb ing a zero order hold element are important parts c the overall dynamic properties of receptor organ investigated by spike sampling procedure (Terzuolo & Knox 1971) This hold property ma well be the combined effect of the encoder action c the receptor cell and the analyzing process in th computer Every hold element consists of two dis tinguishable processes (Elgerd 1967) a samplin process which may here correspond to the spik generation process and the holding property co responding to properties of the analyzing proces By dividing the total transfer function of the re ceptor organ by the transfer function of a zero orde hold element one will obtain separate informatio about the transfer properties of the earlier stages c the signal processing in the receptor organ. Th result of such a division is shown in Fig 6. The dat are those from Fig 5 In the case plotted in Fig 6 the sampling time T of the sample and hold elemen was set equal to 1/13 3 s, a value obtained by roug fitting to the data in Fig. 5 A maximum in the gai curve after subtraction of the hold element is rec ognized This maximum however is found at higher modulation frequency than the maximum fo the total system This difference is not due to th fairly rough estimation of T since the maximum does not move when this value is changed

The gain data of Fig 6 A are plotted in a doubl logarithmic coordinate system The gain seems to increase linearly suggesting a simple power-func tion relation between gain and modulation frequen cy In fitting a power function of the form $y = k \cdot x^n$ to the points in the linear part of the logarithmic plot we found $n = 0.45$ This corresponds to a transfe element of the form $h(s) = s^{n}$

Other investigators (Borsellino et al 1965) hav

Acta Physiol Scand 1980, 109: 447–46..

The pulmonary vasoconstrictor response to hypoxia
The hypoxia-sensitive site studied with a volatile inhibitor

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BJERTNÆS, L., HAUGE, A. & TORGRIMSEN, T. The pulmonary vasoconstrictor response to hypoxia. The hypoxia-sensitive site studied with a volatile inhibitor. Acta Physiol Scand 1980, 109: 447–46.. Received 15 Dec. 1979. ISSN 0001-6772. Institute of Physiology, University of Oslo and Institute of Occupational Health, Ministry of Labour and Municipal Affairs, Oslo, Norway.

Recent investigations have revealed that a number of inhalation anesthetics, including halothane, inhibit the pulmonary vasoconstrictor response to hypoxia without affecting other vasoconstrictor stimuli. Various injectable anesthetics do not show this effect. This discrepancy could be due either to different pharmacological properties or to the different routes of administration. There is no general agreement on whether the response to hypoxia is elicited mainly by airway hypoxia or by blood hypoxemia, or where within the lungs hypoxia acts. This work is an attempt to localize the hypoxia-sensitive site employing halothane. We have studied the reduction of standardized vasoconstrictor responses to hypoxia during administration of halothane via (1) the airways, (2) the pulmonary artery and (3) the pulmonary veins (backward perfusion). Our experimental model has been two pairs of series-perfused hyperventilated isolated rat lungs. An equimolar concentration of halothane most effectively inhibits the response when presented to the alveoli, less when presented to the arterial, and least when presented to the venous segments of the pulmonary vasculature. We suggest that the response to hypoxia is inhibited by halothane at some extravascular site on the arterial side of the pulmonary vasculature, functionally closer to the alveoli than to the responding vessels. A model which combines all the data into an unifying concept has been presented.

Key words: Lung vasoconstriction, vascular resistance, microcirculation, respiration, halothane

Impaired pulmonary gas exchange frequently develops during general anesthesia (Marshall et al 1974). This condition has been suggested in part to be due to inhibition of the pulmonary vasoconstrictor response to hypoxia (Bjertnæs 1978), a mechanism which acts to divert blood from poorly to better oxygenated regions within the lungs (von Euler & Liljestrand 1946).

In isolated rat lungs Bjertnæs (1977) found that pulmonary vasoconstriction in response to hypoxia is reduced in a dose-dependent fashion by several commonly used inhalation anesthetics including halothane. The vascular reactivity to other constrictor stimuli is, however, fully maintained. According to this author no reduction of the vasoconstrictor response to hypoxia occurs following administration of various intravenous anesthetics to the perfusate. This difference in mode of action could be due either to different pharmacological properties of the two groups of anesthetics or to the different routes by which they were administered. It is tempting to link the different effects of the two groups of anesthetics to the question of where within the lungs hypoxia acts. This latter question is much debated (Fishman 1976). Most of the investigators in the field are of the opinion that the response is triggered from a hypoxia-sensi-

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BJERTNÆS, L. HAUGE, A & TORGRIMSEN T The pulmonary vasoconstrictor response to hypoxia The hypoxia-sensitive site studied with volatile inhibitor Acta Physiol Scand 1980 109 447–462 Received 15 Dec 1979 ISSN 0001-677? Institute of Physiology University of Oslo and Institute of Occupational Health, Ministry of Labour and Municipal Affairs Oslo Norway

Recent investigations hav revealed that a number of inhalation anesthetics including halothane inhibit the pulmonary vasoconstrictor response to hypoxia without affecting other vasoconstrictor stimuli Various injectable anesthetics do not show this effect. This discrepancy could be due either to different pharmacological properties or t the different routes of administration. There is no general agreement on whether the response to hypoxia is elicited mainly by airway hypoxia or by blood hypoxemia where within the lungs hypoxia acts. This work is an attempt to localize the hypoxia-sensitive site employing halothane W have studied the reduction of standardized vasoconstrictor responses to hypoxia during administration of halothane in (1) the airways (2) the pulmonary artery and (3) the pulmonary veins (backward perfusion). Our perimental model has been two pairs of series-perfused hyperventilated isolated rat lungs An equimolar concentration of halothane most effectively inhibit the response when presented to the alveoli less when presented to the arterial- and least when presented to the enous segments of the pulmonary vasculature We suggest that the response to hypoxia is inhibited by halothane at some extravascular site on the arterial side of the pulmonary vasculature functionally closer to the alveoli than to the responding essels A model which combines all the data into an unifying concept has been presented

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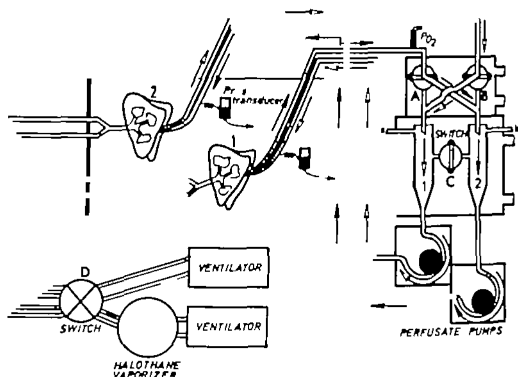


Fig 1 Diagram of the experimental arrangement The isolated rat lung preparations 1 and (inside a thermostated perspex chamber) were perfused with blood by means of two pumps from reservoirs 1 and 2. Effluent blood could b drained back either to the same or to the reservoir of the other preparation (cross-perfusion) via the switches A and B Switch C allowed perfusate overflow at the start of cross-perfusion Pressure transducers were connected to the influent tubings P could be monitored in effluent blood from both preparations. A second cross-connection between preparation 1 and the pump allowed backward perfusion (not shown) Thin catheters were inserted via the side-holes a and b into the inflow tubings for blood sampling. Similar catheters were inserted into the effluent tubings. The preparations were ventilated with two ventilators one connected to a halothane vaporizer Switch D allowed the ventilators to be switched from the one to the other preparation

tive site localized functionally closer to the alveoli than to the blood vessels (Bergofsky et al 1968 Hauge 1969 Hyman & Kadowitz 1975) Others however are in doubt about this alveolar theory (Fishman 1976) They are rather in favour of the view that the response is induced by a direct action of pulmonary arterial hypoxemia on vascular smooth muscle (Campbell et al 1967) If however the concept of alveolar hypoxia as being the main determinant is correct the vasoconstrictor response to a constant hypoxic stimulus should expectedly be more readily damped by a given halothane concentration in the airways as compared to an equimolar concentration in influent blood under condition of low alveolar concentration

The purpose of the present work has been to determine the main hypoxia-sensitive site in the lung with the use of a volatile inhibitor halothane The work can be looked upon as an attempt to test the "alveolar theory"

Instead of comparing the relative effect of alveolar hypoxia and pulmonary arterial hypoxemia we decided to compare the reduction of a standardized vasoconstrictor response by halothane introduced (1) via the airways at low influent blood concentrations and (2) via influent blood at low airway concentrations In some of the preparations a sequence with backward perfusion was included in order to study the effect of pulmonary venous halothane

METHODS

Rats of Wistar strain weighing between 240 and 260 g were anesthetized with diethyl ether When unconscious, pentobarbital 3–4 mg/100 g body weight was injected intraperitoneally

The animals were tracheotomized and continuous positive pressure ventilation wa started keeping end-expiratory pres re at 1.5– .0 cm H_2O A mid-sternal thoracotomy was carried out and 0.5 ml of a solution containing 100 I U pure powdered heparin (Novo) was injected into the left ventricle The heart-lung preparation was removed from the chest and cannulated as previously described (Bjertnæs 1977)

In every experiment, tw lung preparations (no 1 and 2 were simultaneously perfused inside thermostated (37°C) and humidified perspex chamber The perfusate consisted of 35–40 ml of autologous heparinized (10 IU/ml) whole blood obtained by heart puncture of 3–5 donor rats Fig 1 shows the experimental arrangement. Two roller pumps pumped the blood perfusate at constant flow from two reservoirs (1 and 2) into the vasculature of preparations 1 and 2 respectively Until equal perfusate flow had been obtained through both preparations, effluent blood from lung pair 1 was drained back to reservoir 1 and blood from lung pair back to reservoir (independent perfusion). The blood reservoirs, switches and the parts of the tubing lying outside the perspex chamber were also thermostated (37°C)

Perfusion in series ("cross-perfusion) was established by means of the switches A and B The effluent blood from preparation 1 as drained via switch A to reservoir 2 and from preparation 2 via switch B to reservoir 1 In this way the effluent blood from one lung preparation was pumped into the vasculature of the other A third switch (C) was positioned between the reservoirs permitting perfusate overflow at the start of cross-perfusion This switch was closed as soon as both pumps had been adjusted to give exactly the same flow Two series of expts. were carried out In 6 of the xpts of series 1 an additional cross-connection as interposed in the perfusion circuit between preparation no 1 and the pump. This connection was designed in such way that the same lung preparation could be perfused both forward via the pulmonary artery and as backward, via the pulmonary eins The left atrial cannula used in these experiments, was fitted with two side-outlets one attached to an airtrap tubing, the other to pressure transducer Mean pulmonary influent blood pressure was continuously monitored from the pulmonary artery cannula during forward perfusion and from the left atrial cannula during backward perfusion by the use of Statham P 23 Db transducers connected to Sanborn Series 2200 Recorder (Hewlett Packard). Since effluent blood pressure and volume inflow was kept constant each preparation an increase in pulmonary vascular resistance as reflected as proportional increase in influent blood pressure

In some of the expts pulmonary effluent P_{O_2} was measured in both preparations with oxygen electrodes (E 5046, Radiometer Copenhagen) Each P electrode was connected through Physiological Gas Analyzer Model 160 (Beckman) to the Sanborn Recorder

Ventilation The two lung pairs were independently ventilated at frequency of 80 inflations per min. One of the preparations was ventilated with constant volume inflations by the use of Starling Ideal" ventilation pump (C F Palmer Ltd England) T this respirator was connected halothane vaporizer (Drägerwerk, Lubeck, W Germany) The other preparation was ventilated with constant peak pressure inflations by the use of Harvard Apparatus Respirator Between the respirators and the lung preparation (Fig 1) as interposed switch (D), which allowed both the respirator with the vaporizer and that without to be switched from the one preparation to the other

Ventilation as started with 2 3 gentle hyperinflations to peak pressure of 20 cm H_2O which was repeated before each experimental sequence End-e piratory pressure was kept between 1.5 and 2.0 cm H_2O in both preparations by the use of water seals. The respirators were adjusted to give the same peak inflation pressure (8–10 cm H_2O) at the tart of the xperiment. The peak inflation pressure in the preparation which was ventilated w th constant volume inflations was monitored continuously by means of differential pressure transducer (Hewlett Packard Model 220) connected to the Sanborn recorder

I both the e perimental series the t lung pairs were entilated with 5% CO_2 in air (normo ic ga) for period of 8 min which were interrupted by 4 min periods of ventilation with gas containing % O_2 5% CO_2 and 93% N_2 (hypoxic gas) in order to induce asoconstriction.

Minute ventilation was measured with Collins dry spirometer

Serie 1 In these experiments both preparations were simultaneously entilated with the hypoxic gas mi ture Since the preparations were perfused in series, the stimulus for vasoconstriction w combination of alveolar hypoxia and influent hypoxemia.

Serie 2 I these xperiments one of the preparations was entilated with the hypoxic the other with the normoxic gas Since the lung pairs were series-perfused the preparation ventilated with the hypoxic gas was perfused with blood with high PaO_2, the stimulus for as oconstriction in these preparations was alveolar hypoxia only The term influent blood used in this article is identical with pulmonary arterial blood during forward perfusion and pulmonary venous blood during backward perfusion Correspondingly the term effluent blood means the blood coming from the lung vasculature irrespective of flow direction.

Determination of halothane in blood Samples containing 200 µl of blood were draw with micropipette from effluent blood during halothane administration via the airways and from both influent and effluent blood during administration via the bloodstream The blood samples were taken via small catheters one inserted into the left atrial tubing the other nto the pulmonary arterial tubing via side holes in the reservoirs (and *b*) (Fig. 1) The blood specimen was diluted with 0 5 ml of water containing N-butanol as nternal standard and sodium lauryl sulphate 1% as surfactant in 1 ml mocrovial properly sealed with mininert valves (Supelco Inc) A volume of 1 µl of this solution was njected into Perkin Elmer F 11 gas-chromatograph equipped with flame ionization detector and 6 ft glasscolumn packed with Chromosorb 102 and precolumn of teflonpipe Injection temperature was 150°C oven and detector were kept at 100 and 200°C respectively

Standard solutions of halothane were prepared in water and peak height ratios were used for quantitative determinations. The peak height ratios were reduced according to the internal standard. In order to reduce possible concentration gradients, the samples were irradiated for tw minutes in an ultrasonic bath before injection At least 3 injections were carried out from each probe

Determination of end-expiratory halothane concentrations In 8 preparations of series 1 end-expiratory halothane concentrations were determined with Cen-

tronic mass spectrometer (0th Century Electronics Ltd England) and recorded on a W+W recorder Model 314 (W+W Electronic Inc Switzerland) Respiration gas was continuously sucked into the mass spectrometer at a flow rate of 4 ml/min through a cannula positioned in the trachea The mass spectrometer was calibrated for concentrations of halothane ranging between 0 and 5% by means of the halothane vaporizer which again had been controlled by the use of a Riken gas indicator (Riken Koiki Instruments Co Japan) In addition concentrations of halothane between 0 and 5% from the vaporizer were equilibrated at an air/halothane flow of 1 5 l/min with thermostated (37°C) water in a 100 ml impinger bottle with a glass frit (Bistabil NS 29/3 W Germany) Equilibrium was judged by the occurrence of equal halothane concentrations in the gas to and from the bottle The halothane water concentration corresponding to 1% halothane in air was 5 98 mg/100 ml Based on partition coefficients between blood and gas and water and gas of 2.36 v/v % and 0 77 v/v % (Lauven et al 1979) respectively the corresponding blood concentration should be 18.35 mg/100 ml The theoretical blood concentration corresponding to a gas concentration of 1% is 18 31 mg/100 ml as calculated on the basis of the molecular weight of halothane being 197

Determination of pulmonary shunt flow In 6 of the preparations of series I the pulmonary shunt fraction Qs/Qt was determined using the formula

$$Qs/Qt = \frac{Cc\ Hal - CaHal}{Cc\ Hal - C\bar{v}Hal}$$

The end-capillary halothane concentration Cc Hal was calculated on the basis of the end expiratory concentration mea ured with the ma spectrometer (1% = 18 31 mg/100 ml) CaHal and C$\bar{v}$Hal are the halothane concentrations in pulmonary effluent and influent blood respectively Qs/Qt was calculated following administration of halothane both via the airways and via blood

Statistics Unless another method is indicated Wilcoxon two-sample test (two-sided) wa used The statistical evaluations were carried out by means of a computer programme for statistical routines on Nord 10 (Norsk Data A/S)

RESULTS

After having established series perfusion ("cross-perfusion) of two lung pairs standardized pulmonary vasoconstrictor responses to ventilation hypoxia (F_{I} =0 02) were elicited as previously described (Hauge 1968 Bjertnæs 1977) Since flow was kept constant in each preparation changes in pulmonary arterial pressure directly reflected changes in vascular resistance A reversible rise in pulmonary arterial pressure due to hypoxia is traditionally called a pressor response Before any pharmacological interference two control responses of equal or slightly increasing magnitude were elicited Halothane was administered from the point of termination of the last of the control responses to the cessation of the next period of hypoxia

Median minute ventilation in 3 lung pairs was 8[illegible] ml (range 78–84 ml) at peak inflation—and end-expiratory pessures of 10 and 2 cm H_2O respectively

Series I Effects of halothane on the response to combined alveolar and influent blood hypoxia

Fig 2 demonstrates typical pressor responses to combined alveolar and influent blood hypoxia and the concomitant reductions in pulmonary effluent blood P in one pair of isolated lungs (Exp 8 prep 1) The upper panel shows two control responses followed by a test response which was reduced during administration of halothane via the pulmonary artery In the next sequence (lower panel) the anesthetic was administered via the airways On this occasion the response almost disappeared Fig 3 demonstrates from two typical experiments the relationship between percentual reduction of the pulmonary pressor response to hypoxia and the corresponding blood concentrations of halothane following both routes of administration Open circles symbolize response reduction to halothane administered via the airways plotted against the resulting venous effluent blood concentrations The latter values normally reflect the level of alveolar concentrations Closed circles indicate response reduction during pulmonary arterial administration plotted against the concentrations in influent blood Effluent blood concentrations are given in the parenthesis Continuous lines combine results obtained in the same lungs Ideal experimental conditions as regards the efficiency of alveolar ventilation are nearly obtained in the preparation presented to the left (Fig 3) In this preparation (Exp 3 prep 1) the response was completely abolished during halothane administration via the airways giving an effluent blood concentration of 22 mg/100 ml The influent blood concentration on this occasion was only 1 9 mg/100 ml due to effective elimination of halothane via the other lung pair In comparison halothane in a concentration of 18 mg/100 ml administered via the pulmonary artery resulted in a 15% reduction of the response only In this case effluent blood halothane concentration was as low as 2 3 mg/100 ml reflecting a very efficient "dehalothanization of blood

In several preparations however considerably

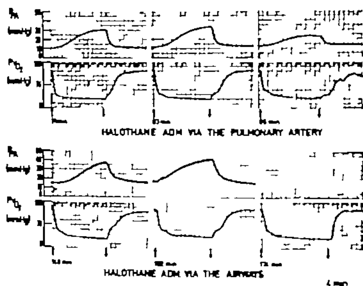


Fig 2 Pulmonary pressor responses to hypoxia in one pair of isolated rat lungs before and during administration of halothane via the pulmonary artery (upper panel) and via the airways (lower panel) Concomitant reductions in effluent blood P are shown below the pressure tracings. Arrows indicate start and termination of hypoxic periods. Time from start of perfusion is given.

higher effluent blood concentrations were seen during administration via the pulmonary artery although the pressor response was fairly well maintained For instance, in the preparation presented to the right in Fig 3 which is identical with that shown in Fig. 2 the influent blood concentration of halothane was 88 mg/100 ml. The corresponding value in effluent blood was 20 mg/100 ml The reason for this relatively high effluent blood concentration is outlined below In spite of the high halothane concentration in influent blood however the response was not reduced by more than 50 % In contrast, airway administration resulting in a concentration of 77 mg/100 ml in effluent blood, completely prevented the response Fig. 4 is similar plot, including all the expts of this type It is evident from this diagram that arterial (closed circles) is less efficient than alveolar administration Table 1 gives a complete survey of the results obtained in 33 of a total number of 4 lung pairs. Results from the remainder of the preparations were discarded due to spontaneously falling control responses or the absence of any response at all Perfusate flow baseline inflow pressure (P) the mean of two control responses (ΔP mean) and the magnitude of the response during halothane administration (ΔP) is tabulated, as well as the percentual response reduction and the blood concentrations of halothane

Administration of halothane via the airways resulting in a median effluent blood concentration of 33 mg/100 ml (range 10–88 mg/100 ml) reduced the response with a median of 100 % (range 71–100 %) I comparison, a median reduction of 50 % (range 0–79 %) was obtained during administration of the anesthetic via the pulmonary artery giving median influent and effluent blood concentrations of 36 mg/100 ml (range 18–88 mg/100 ml) and 1 mg/100 ml (range –31 mg/100 ml) respectively The difference between the concentrations of halothane is primarily a measure of alveolar ventilation in these preparation

Median perfusate P_{O_2} in 8 of the expts was 120 mmHg (range 110–128 mmHg) during ventilation with the normoxic gas and 5 5 mmHg (range 15–30 mmHg) during ventilation with the hypoxic gas.

In 6 expts the effect of halothane administered in the pulmonary veins was studied during a period of backward perfusion preceeding administrations via the airways and via the pulmonary artery Table 2 lists the relevant data from this test sequence The preparations can be identified by their individual expt and prep nos given in Table 1 Fig. 5 shows tracings of the pulmonary influent blood pressure

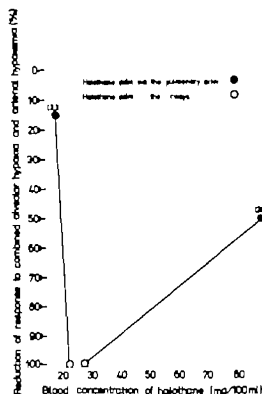


Fig 3 Reduction of pressor responses to hypoxia of halothane administered via the pulmonary artery and via the airways plotted against concentrations of the anesthetic in influent and effluent blood respectively Lines connect tests in the same rat lung preparation Numbers in parenthesis indicate effluent blood concentrations during arterial administration In one of the preparations the latter concentration was low in the other high

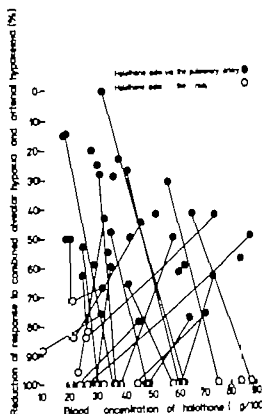


Fig 4 Reduction of pressor responses to hypoxia in rat lungs of halothane administered via the pulmonary artery and via the airways plotted again t the resulting concentrations in influent and effluent blood respectively Lines combine tests within the same preparations

and the effluent blood P from a typical preparation (Expt ?0 prep 1) The responses demonstrated in the upper panel were obtained during backward perfusion The two lower panels show tracings obtained during normal anterograde perfusion During administration of halothane via the pulmonary veins resulting in concentrations of 37 mg/100 ml in influent blood and 1? mg/100 ml in effluent blood the pressor response (upper panel) was reduced by 9% Administration of the anesthetic via the pulmonary artery (middle panel) at concentrations in influent and effluent blood of 33 and 10 mg/100 ml respectively reduced the response with 43% Finally halothane was administered via the airways resulting in a concentration of 23 5 mg/100 ml in effluent blood which reduced the response with 93%

Fig 6 presents the relationship between reduction of response and blood concentration of halothane following administration of anesthetic via all the three routes Reductions caused by venous (semiclosed circles) and arterial (closed circles) administration respectively are plotted against the halothane concentrations in pulmonary influent blood Simultaneously determined concentrations in effluent blood are shown in the parenthesis Reductions caused by airway administration (open circles) are plotted against the resulting concentrations in effluent blood In each lung preparation three levels of response reduction was found depending on whether halothane was given via the veins the artery or the airways Halothane administered via the pulmonary veins reduced the response with a median of 23 5% (range 8 3–62%) The median influent and effluent blood concentrations at this stage were 4? mg/100 ml (range 37–81 mg/100 ml) and 15 5 mg/100 ml (range 6–22 mg/100 ml) respectively In comparison a median reduction of 58 5% (range 4?–79%) was observed during pulmonary arterial administration resulting in median influent and effluent blood concentrations of 39 mg/100 ml (range 3?–75 mg/100 ml) and 11 5 mg/100 ml (range 10–18 mg/100 ml) respectively Airway administration reduced the re-

sponse with a median of 90.5% (range 71–100%), the median effluent blood concentration on this occasion being 23.5 mg/100 ml (range 10–39 mg/100 ml).

The damping effect of halothane following all the three routes of administration, could now be compared by using the ratio between reduction of response and blood concentration of anesthetic ("damping ratio"). The results are given in Table 3. Evaluation of the results with the Wilcoxon paired sign test (two-sided) shows that the ratio between percentual reduction of pressor response and influent blood concentrations of halothane is significantly smaller when the anesthetic is administered via the pulmonary veins as compared to administration via the pulmonary artery ($P = 0.03$). Furthermore, this latter ratio is significantly smaller than the ratio between the percentual reduction of response and effluent blood concentration resulting from airway administration of halothane ($P = 0.03$).

The median ratio between percentual reduction of response and effluent blood concentration of halothane during pulmonary arterial administration (6.3%/mg/100 ml), is higher than the similar ratio obtained during airway administration. This difference could be due to a combined effect of halothane near or in the wall of the pulmonary arteries and near the alveoli. If, however, one considers a single hypoxia-sensitive site only, an equimolar concentration of halothane most effectively inhibits the pulmonary pressor response to hypoxia if presented to the alveoli, less if presented to the pulmonary arterial segments and least if presented to the venous segments.

During administration of halothane via the blood, surprisingly high effluent blood concentrations were observed in some of the preparations. We therefore calculated the shunt flow fraction (Qs/Qt). Table 4 gives a survey of the 8 preparations examined. The individual lung pairs can be identified by their expt. no. and prep. no. as referred to in Table 1. Concentrations of halothane in pulmonary influent, effluent and end-capillary blood are tabulated. The latter values were calculated on the basis of end-expiratory halothane concentrations determined with mass-spectrometer. Qs/Qt was calculated at an early stage of the experiment, and then upon one or two times during test responses. There was a tendency for Qs/Qt to increase from the first to the second or third measurement. For instance in expt. 14, prep. 1, Qs/Qt increased from 0.03 shortly after the start of perfusion to 0.74 during a test response during exposure to halothane introduced via the pulmonary artery. In one of the preparations (expt. 1, prep. 1) Qs/Qt was determined also during retrograde perfusion.

A certain loss of halothane from tubings and reservoirs might be expected. The reduction in halothane blood concentration due to such loss was 10.3% (mean) from the effluent side of the lung pair being exposed via the airways to the influent side of the lungs exposed via blood.

Series 2. Effects of halothane on the response to alveolar hypoxia

In 10 experiments the period of ventilation hypoxia was confined to one of the lung pairs only, while the other preparation was ventilated with normoxic gas. The median influent P_{O_2} during ventilation hypoxia in 4 of the test lung preparations was 118 mmHg (range 114–128 mmHg), whereas the simultaneously determined effluent blood P_{O_2} was 35 mmHg (range 22–42 mmHg). In spite of high influent blood P_{O_2}, ventilation hypoxia induced pressor responses, which were on the average only 13% smaller than those obtained in series 1. Fig. 7 shows pulmonary arterial pressure tracings from one typical preparation (expt. 28, prep. 2). Halothane administered via the airways resulting in an effluent blood concentration of 28 mg/100 ml reduced the response with 100% (upper tracing). In contrast, a pulmonary arterial halothane concentration of 70 mg/100 ml, reduced the response with 27% only (lower tracings). The corresponding effluent blood concentration was 5.5 mg/100 ml.

When halothane had been administered both via the pulmonary artery and via the airways in one of the two series-perfused preparations, the same procedure was repeated with the other one. Table 5 lists the complete results. Halothane was administered via both routes in a total of 10 pair of lungs. In 15 preparations administration was limited to one route only. The median reduction of the pressor response was 62.5 (range 0–82%) during halothane administration via the pulmonary artery, giving a median influent blood concentration of 53 mg/100 ml (range 11–84 mg/100 ml). The simultaneously determined median concentration in pulmonary effluent blood, reflecting the efficiency of alveolar ventilation, was 14 mg/100 ml (range 3–34 mg/100 ml). Following administration of halothane via the airways, median reduction of response was 100%

Table 1 *Comparison of the effect of pulmonary arterial and airway administration of halothane on pulmonary pressor response to combined alveolar hypoxia and arterial hypoxemia*

			Halothane administration via the pulmonary artery						Halothane administ	
				Responses before halothane adm	Response during adm of halothane		Blood concentration of halothane mg/100 ml			Response before halothane adm.
Exp no	Lung no	Flow (ml/min)	Base line P (mmHg)	ΔP mean (mmHg)	ΔP_m	% reduction	Pulm influent	Pulm effluent	Base line P (mmHg)	ΔP mea (mmHg)
1	1	20	17	[illegible]	6	63	[illegible]5 0	16 0	19	22
	2	20	16	22	9	59	[illegible]9 0	7 0		
[illegible]	1	20	11	14 5	8	45	46 0	8.0	10	31.5
	1	[illegible]0	16	18	9	[illegible]0	19.2	10 0		
3	1	15	10	13	11	15	18 0	2.3	13	1[illegible]5
	2	15	17	10	5	50	[illegible]0 0	[illegible]3	10	11
4	1	16	11	1	8.5	[illegible]9	36 0	4 1		
5	1	14	9	13	10	[illegible]3	38 0	8.0		
6	1	18	16	10	5	[illegible]0	4 0	14 0		
	1	18	19	9	4	55.5	34 0	14 0		
		18	12	12	5	58	84 0	[illegible]0 0	1	21
7	1	15	13	8.5	4	53	25 0	14 0	15	10.5
	2								1	1
8	1	[illegible]0	11	1	11.5	50	88 0	[illegible]0 0	17	4
	1	20							16	15.5
	2	[illegible]0	[illegible]0	[illegible] 5	9	60	62 0	31 0		
9	1	15	1	1 5	10	[illegible]0	[illegible]8 0	7 0		
	1	15	14	21	5	76	[illegible]0 0	17 0	15	16
		15	19	1[illegible]5	5	60	35 0	4 0	13	12
10	1	16	10	18 5	13	7	41 0	3.5	15	16
	2	16	15	22	15	31	56 0	3.5	15	26.5
11	1	16	18	[illegible]6	15	42	65 1	19 2	16	20
12	1	18	15	17	6	64	73 6	21.3	17	22
13	1	18	14	[illegible]3 5	8	66	41	18.2	12	17.5
		18	14	23	5	78	64 0	24 9	15	25
14	1	16	1	18	13	28	31 0	[illegible]3 0	17	27
15	1	18	10	33	19	42	51 0	1 0		
	2	18	18	28	21	25	30	16.2		
16	1	10	10	19	9 5	50	58 0	1[illegible]0	1[illegible]5	[illegible]5
		10	4	[illegible]3	1	48	35 0	10 0	4	14
17	1	10	10	14	3	79	4[illegible] 0	12 0	14	10
		10	6	10 5	9	14	19 0	3 0	6	10
18	1	12	16	15	5	67	32 0	11 0	[illegible]5	4
	2	1	16	20	1	0	3 0	5 0	10	17
19	1	16	12	17	4	76	3 0	11 0	15	17
20	1	18	19	21	1	43	33 0	10 0	20	[illegible]9
1	1	13	11.5	19	11	42	75 0	18 0	15	[illegible]5

(range 88–100%) which was obtained with a median effluent blood concentration of [illegible]9 mg/100 ml (range [illegible]4–56 mg/100 ml) Fig 8 illustrates the reduction in response during administration of halothane via the pulmonary artery (closed circles) and via the airways (open circles) plotted against the concentrations of anesthetic in pulmonary influent and effluent blood respectively Test points from the same preparations are connected by continuous lines Statistical evaluation of the results shows that the ratio between reduction of response and effluent blood concentration of halothane following administration via the airways (medi 3 45%/mg/100 ml (range 1 79–4 17%/mg/100 ml is significantly higher ($P<0\ 001$) than the ratio b tween the reduction of response and the influe blood concentration of halothane (median 1 04 mg/100 ml (range 0 002–2 [illegible]1%/mg/100 ml)) o served during pulmonary arterial administration.

DISCUSSION

A striking result of the present expts was th equimolar concentrations of halothane reduced th

le arvay

[illegible] reduction	Pulm effluent blood concentration of halothane (mg/100 ml)
12	77
14	22
100	22
100	21
100	14
100	31
100	33
100	77
100	30
100	45
100	30
100	60
100	75
100	[illegible] 6
100	61.6
100	58 3
100	48 [illegible]
100	37 [illegible]
100	39 0
100	40 0
100	4 0
100	37 0
71	21 [illegible]
100	6 [illegible]
88	10 0
93	23 5
84	26 0

response more effectively when presented to the airways than when presented to the blood When administration via the blood is considered more effective inhibition was obtained when halothane was presented in equimolar concentrations to the arteries than when presented to the veins.

There are several advantages in making use of an inhalation anesthetic to study the location of the hypoxia-sensitive sit within the lungs. (1) The inhibition of the pressor response to hypoxia is reversible and specific (Bjertnæs 1977) (2) A volatile inhibitor can be administered and eliminated by lung ventilation thereby allowing the effect to be studied within the same preparation during administration via d fferent routes (3) Halothane is dissolved in blood in proportion to its blood/gas partition coefficient End-capillary partial pressure of the anesthetic is equal to the al eolar partial pressure (Nunn 1969) in lungs with no or very small shunt flow fractions.

During blood adm nistration of the anesthetic attempts were made to keep the effluent blood- and consequently also the alveolar concentration as low as possible by hyperventilation of the lungs. In this way the effect of halothane on the response could be confined to a ascular segment positioned upstreams to the alveoli i e an arterial segment dur ing forward perfusion and a enous segment during backward perfusion Likewise a low influent blood concentration was aimed at during airway administration

The result of the two series of expts will be discussed together Although combined hypoxic stimulation (arterial+al eolar) ga e somewhat larger responses probably due to the lower perfusate P_{O_2}, no ystematic differences were observed concerning the effects of halothane

A close examination of Tables 1 and 5 reveals that during *pulmonary rterial admi stration* of halothane the low effluent blood concentration aimed at was obtained in about one fourth of the preparations (both preparations in expts 3 and 10 preparation 1 in expts. 4 13 and 25 and preparation in expts 17 18 and 28) In most of these preparations the damping effect of halothane wa small In expts 18 and 25 preparations and 1 no response reduction occurred at all in pite of influent blood halothane concentrations of 37 and 30 mg/100 ml respectively In all the above lung preparations, the high concentrations of halothane must have been almost completely limited to the arterial segment and the contribution to response reduction caused by halothane in othe lung regions negligible

During *irway administration* the response in most of the preparations disappeared already when the effluent blood halothane concentration was between 20 and 30 mg/100 ml In some preparations however much higher effluent concentrations were induced, the purpose being to establish a high influent blood concentration in the other pair of series-perfused lungs

Let us now focus on the preparations in which halothane had been adm nistered successively via

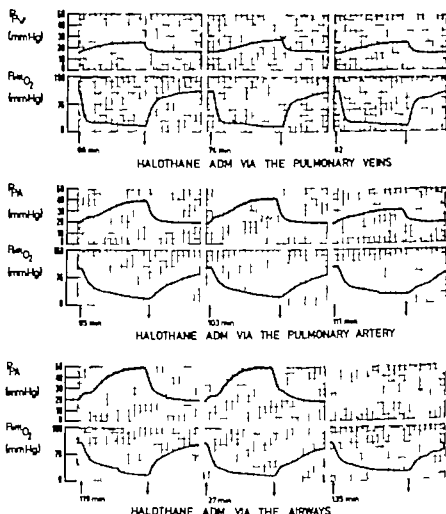


Fig 5 Pulmonary pressor responses to hypoxia in one pair of isolated rat lungs before and during administration of halothane via the pulmonary veins (upper panel) the pulmonary artery (middle panel) and the airways (lower panel) Concomitant reductions in effluent blood P_{O_2} are shown below the pressure tracings Arrows indicate start and termination of hypoxic periods. Time from start of perfusion is given Flow was constant (18 ml/min) regardless of flow direction

all the three routes Table 1 and 2 During *venous administration* the response was reduced with only 23.5% (median) in spite of an influent halothane blood concentration of 4? mg/100 ml (median) The corresponding effluent i e the pulmonary arterial concentration was then 15 5 mg/100 ml (median) When in contrast halothane was administered *via the pulmonary artery* resulting in approximately 2 5 times the latter concentration response reduction also increased ?.5 times The alveolar concentrations on these occasions as reflected by the effluent blood concentrations were about the same (in medians 15 5 and 11.5 mg/100 ml during pulmonary venous and arterial administration respectively) These observations strongly indicate that when the alveolar concentration is constant the reduction of the pressor response to hypoxia is determined by the pulmonary arterial and not by the venous halothane concentration A further implication is that the hypoxia sensitive region in the lungs is located functionally and probably also anatomically closer to the arterial than to the venous segment of the vasculature This latter interpretation is consistent with the demonstration of mainly precapillary vasoconstriction in response to hypoxia in intact cat lungs (Kato & Staub 1966) and in isolated lungs from rats (Aarseth et al 1980)

The damping effect of equimolar concentrations of halothane during administration via different routes could be compared directly by calculating the ratios between response reduction and the halothane concentrations in the different compar

Table The effect on the pulmonary pressor response to combined blood/alveolar hypoxia of halothane administered in the pulmonary vein

Exp. no.	Lung no.	Flow (ml/min)	Base line P_{PA} (mmHg)	Responses before halothane adm. ΔP mean (mmHg)	Response during adm. of halothane ΔP_{PA}	% reduction	Blood concentration of halothane mg/100 ml Pulm. influent	Pulm. effluent
[illegible]	1	10	9	1	11	8.3	48 0	10 0
[illegible]	1	10	4	11	7	36	37 0	19 0
[illegible]	1	12	13	13	5	6_	46 0	21.0
[illegible]	1	16	10	15	9	40	38 0	6.0
[illegible]	1	18	15	11	10	9	37.0	1 0
[illegible]	1	13	13	9	8	11	81 0	22.0

...tests. These damping ratios obtained during pulmonary arterial and venous administration (Table 3), were only 42 and 16% respectively of that resulting from administration via the airways. The highest ratio was, however, found between response reduction and effluent blood concentration during administration of halothane via the pulmonary artery. It is a striking result that the difference between this latter ratio (5.20 %/mg/100 ml) and that resulting from airway administration (3.67 %/mg/100 ml) is identical to the ratio between response reduction and influent blood concentration obtained during arterial administration (1.53 %/mg/100 ml). Just as combined arterial and alveolar hypoxia is a more potent vasoconstrictor stimulus than alveolar hypoxia alone, so is a combined arterial and alveolar halothane administration a more efficient way to inhibit the response than alveolar administration alone. If we make the assumption that the ratios are additive the reduction of the pressor response to hypoxia during arterial administration represents 29% of the total and the alveolar administration 71%. These findings are consistent with the earlier observations by Hauge (1969) that alveolar hypoxia and not arterial hypoxemia is the main denominator for the pulmonary pressor response to hypoxia. H found that the two types of stimuli interact in a way depending upon the relative intensity of ventilation and perfusion.

In a recent study also carried out in series-perfused rat lungs Bjertnæs & Mundal (1980) showed that the inhalation anesthetic enflurane during administration via the airways reduced the pressor response to hypoxia in a fashion depending on the end-expiratory enflurane concentration (determined by mass-spectrometry).

Since in the present study the ventilation/perfusion relationship was consistently high (4 to 8) it could be argued that more halothane is delivered by the airways than by blood in spite of the fact that

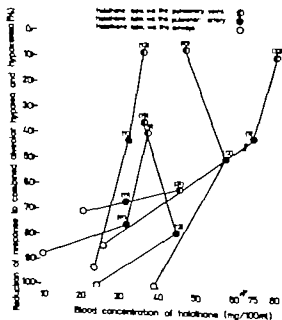


Fig. 6. Reduction of the pressor response to hypoxia of halothane administered via the pulmonary veins and artery plotted against influent blood concentrations and via the airways plotted against effluent blood concentrations of the anesthetic. Lines connect tests within the same isolated rat lung preparation. Numbers in parentheses indicate effluent blood concentrations during administration via blood.

Table 3 *Median ratios between reduction of response to hypoxia (%) and blood concentration of halotha (mg/100 ml) during administration via three different routes*

Pulmonary veins	Pulmonary artery	Airways
0 60 %/mg/100 ml (range 0 14–1.35)	1.53 %/mg/100 ml (range 0.56–[illegible].38)	3 67 %/mg/100 ml (range [illegible].56–8.80)

During airway administration the ratio was calculated on the basis of the effluent blood concentration whereas influ blood concentrations were used for the other ratios

the latter concentration in most cases was higher This could then possibly explain the route-dependent difference in damping effect If however the amount of halothane and not the partial pressure (proportional to the concentration) is the critical factor the largest response reductions should expectedly occur in the preparations with the highest blood flow and particularly in those with a combination of high influent concentration and high flow Such combination was present in both preparations in expts 1 ? and 8 The reduction of the pressor response observed in these preparations was however not larger than that seen in preparations with much lower blood flows Ventilation w about the same in all preparations In a study co cerning the ability of alveolar hypoxia versus a terial or venous hypoxemia to elicit pulmonary vas constriction Bergofsky et al (1968) found that t response was mainly determined by alveolar P irrespective of whether the ventilation/perfusion r lationship had been 1.5 or 5 The response obtain during pulmonary venous or postcapillary h poxemia was negligible

The high effluent blood concentration of H lothane observed in some of the preparations du ing administration via blood was found to

Table 4 *Calculated fraction of right to-left shunt (Q /Q) in isolated perfused rat lungs*

Exp no	Prep no	End Expir halothane conc (%)	Halothane blood concentration (mg/100 ml) End-capillary[a]	Pulmonary influent	Pulmonary effluent	Q/Q
11	1	4 90	89 7?	3? 70	88 60	0 0?
11	1	0 70	?3 66	65 10	19 ?0	0.25
12	1	4 40	80.56	21.30	79 90	0 01
12	1	0 ?5	4 60	60 ?	17 80	0 ?4
13	1	3 80	69 58	4 90	58.30	0 ?5
13	1	0 70	3 66	41 ?0	18.20	0 39
13	2	5 00	91 55	? 10	87 30	0 07[b]
13		0 35	6 41	64 00	24 90	0 3
14	1	5 00	91 55	4 00	89 80	0 03
14	1	0 39	7 14	74 90	77.80	0.30
14	1	0 10	1 83	31 00	23 00	0 74
15	1	5.20	95.?1	19 ?0	84 80	0 14[b]
15	1	0 40	7 3	87 40	19 00	0 15
15	1	0 30	5 90	51 00	1 00	0 34
15		5 30	97 04	19 00	97 80	0 05
15		0 30	5.50	85 00	19 20	0 17
15	2	0 15	75	30 ?0	16 20	0 49
1	1	5 00	91.55	22 10	87 30	0 07[b]
1	1	0 16	2 93	81 00	22 10	0 25
1	1	0 16	2 93	74 80	18 30	0.21

Calculated on the basis of measured end-expiratory halothane concentrations
Results obtained during airway administration of halothane just after start of perfusion The other result obtained during influent blood administration of the anesthetic
Result obtained during retrograde perfusion.

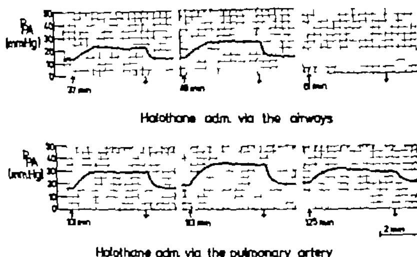


Fig. 7. Pulmonary pressor responses to alveolar hypoxia in one part of isolated rat lungs before and during administration of halothane via the airways and via the pulmonary artery. Arrows indicate start and termination of ventilation hypoxia. Time from start of perfusion is given as well. In contrast to the situations illustrated in Figs. 2 and 5 influent blood P_{O_2} was kept high (above 114 mmHg).

...ved by a high calculated shunt flow fraction which tended to increase during perfusion. Whether the calculated shunt developed because of some undefined deterioration of the lungs or because of a changing ventilation/perfusion relationship due to hypoxia-induced vasoconstriction per se is not known. Histological examination of isolated rat lung preparations ventilated and perfused for 3 h, showed scattered regional differences in lung structure, some regions being atelectatic, others being hyperinflated (Bjertnæs et al. 1980). The calculated shunt flow could also be caused by uneven distribution of vasoconstriction during hypoxia resulting in decreased perfusion in some regions and overperfusion and high vascular pressures leading to edema development in others (Hultgren & ... 1966). Whether the shunt-like effect as observed, is due to true shunting of blood or to mismatching of ventilation and perfusion is not known. Since the shunt was examined with a tracer gas with a very low partial pressure the shunt as measured is probably overestimated, most of it being due to ventilation/perfusion mismatching.

In the preparations with ventilation/perfusion ab-

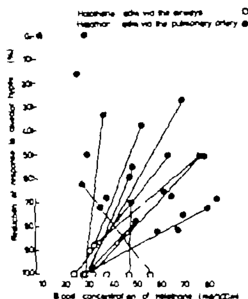


Fig. 8. Reduction of pressor responses to alveolar hypoxia (no influent blood hypoxemia) in rat lungs of halothane administered via the pulmonary artery and via the airways, plotted against the resulting concentrations in influent and effluent blood, respectively. Lines combine tests within the same preparations.

Table 5 *Comparison of the effect of pulmonary arterial and airway administration of halothane on pulmonary pressor response to alveolar hypoxia*

			Halothane administration via the pulmonary artery						Halothane administr[illegible]	
				Responses before halothane adm	Response during adm of halothane		Blood concentration of halothane mg/100 ml			Responses before halothane adm
Exp no	Lung no	Flow (ml/min)	Base line P (mmHg)	ΔP mean (mmHg)	ΔP_m	% reduction	Pulm influent	Pulm. effluent	Base line P (mmHg)	ΔP mean (mmHg)
22	1	15	[illegible]0	1[illegible]	3	75	70 0	8.0		
[illegible]3	1	15	15	1	6	50	30 0	4 0		
4	1	10	[illegible]7	15	5	67	38.0	14 0		
24	2	10	2[illegible]	18.5	5	7[illegible]	35 0	14 0		
25	1	14	14	11	11	0	30 0	7 0		
25	1	14	18	11	3	82	59 0	14 0		
25	2	14	[illegible]0	[illegible]	9	59	48 0	1 0		
6	1	1	19	18	5	7[illegible]	80 0	14 0	4	[illegible]3
26		12							[illegible]0	[illegible]3
27	1	10	14	8	3	6[illegible]5	28 0	15 0	14	7.5
27	2	10	17	10	3	70	48 0	7 0	10	10
28	1	14	1	22	5	77	50 0	16 0	11	11
28	2	14	16	15	1	[illegible]7	70 0	5 5	14	10
28		14	18	14	7	50	79 0	7 0		
28	2	14	4	17	19	0	11 0	3 0		
29	1	1	1	9	4	55	49 0	9 0	15	10
30	1	14	18	20 5	10 [illegible]5	50	78.0	4 0	1	20
30	1	14	25	[illegible]7	5	81	68 0	34 0		
30		14	18	1	6	50	64 0	31 0	16	11
30		14	30	1	4	67	65 0	4.5		
30	1	14	[illegible]5	[illegible]5	1	16	26 0	18.0		
31	1	14	14	16	10	37 5	53 0	15 0	11	9
31	1	14	[illegible]0	14	4 5	68	84 0	8.0		
31	2	14	19	15	10	33	37 0	16 0	18	9
31	2	14	24	13	4 5	65	6[illegible]0	8 0		

normalities the end-expiratory halothane concentrations measured as concentrations in effluent blood might have been underestimated This does not however appear to be the case Mass spectrometrical measurements of end-expiratory halothane concentrations during airway administration in 8 preparations (Table 2) differed only slightly from those determined in effluent blood with gas chromatography

Bearing in mind that small muscular arteries at the level of the terminal or respiratory bronchioles constrict in response to alveolar hypoxia (Kato & Staub 1966) it was not surprising to find that the response was least reduced when halothane was administered via the pulmonary veins Thus the site from where the vasoconstrictor response to hypoxia is elicited must also be localized on the arterial side of the pulmonary vasculature Consequently blood should expectedly be more effectively "dehalothanized" before the hypoxia-sensi[illegible] tive region is reached during pulmonary venous [illegible] than during arterial administration

How can we explain the different susceptibility [illegible] halothane depending on whether the anesthetic [illegible] administered via the airways or via the pulmonary [illegible] artery? Unless we know the profile of the halothane [illegible] partial pressure curves from alveoli to the core of [illegible] the responding vessels no exact answer can be [illegible] given Such data are non-existent An answer can [illegible] however be approximated by extrapolating from [illegible] information about oxygen diffusion from alveoli [illegible] small pulmonary arteries in lungs from cats ventil[illegible] lated with oxygen prior to rapid freezing (Staub [illegible] 1961) In arteries with diameters below [illegible]00 μ an[illegible]

he airway

...ponses during ...n of halothane % reduction	Pulm. effluent blood concentration halothane (mg/100 ml)
100	28 0
100	38 0
100	56 0
100	47.0
100	28 0
100	28 0
100	29 0
98	30 5
100	4 0
88	31.0
100	28 0

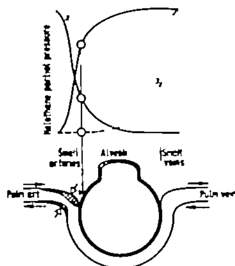


Fig 9 The lower panel shows schematic lung model with the proposed hypoxia-sensitive site ( ) on the arterial side of the asculature The upper panel show hypothetical halothane partial pressure curves along the pulmonary vasculature during administration via the air ways (1) the pulmonary artery (?) and the pulmonary veins (3) The open circles symbolize the projection of the hypoxia-sensitive sit on the halothane partial pressure curves, thereby indicating the assumed relation between the halothane partial pressures at this site during administration via the different routes. Continuous and broken arrows indicate flow direction whereas small arrows ind' cate the proposed vasoconstrictor site. Fig. 6 should be studied simultaneously

outer zone of bright red blood was found whereas the vessel core was till magenta. Mainly on the basis of this observation Hauge (1969) proposed a flat oxygen tension gradient from alveolar wall to vessel lumen, and steep in blood interface With improved technique Conhaim & Staub (1979) recently confirmed that 51% of pulmonary arteries with diameters between 475 and 200 μ taken from oxygen-ventilated cat had an outer rim of blood whose oxygen saturation was 30% above that of the vessel core The oxygen saturation of the other small arteries corresponded to alveolar P_{O_2} i.e. the P_{O_2} gradient from alveoli to the core of these vessel was almost quite flat.

In the present work, the response t hypoxia was more effectively damped during airway- than during blood administration halothane Such result i hard to conceive in the presence of a flat halothane tension curve from the alveoli to the core of the responding vessels With a flat, or almost flat curve the response should expectedly ha e been reduced by the same amount irrespective of airway—or blood administration The marked difference re lated to the route of administration can however be explained by postulating a halothane gradient similar to that originally proposed for oxygen (Hauge 1969)

Fig. 9 shows hypothetical curves for the changes in halothane tension along the pulmonary vasculature during administration via the airw ys (1) the pulmonary artery ( ) and the pulmonary vein (3) Below is schematic model of the lung. The continuous and broken arrows indicate forward and backward perfusion respectively whereas the short arrows indicate the supposed site of vasoconstriction. Since the response to hypoxia is damped by halothane in dose-dependent fashion (Bjertnaes

1977) the concentration of anesthetic at the hypoxia-sensitive site (circles in Fig 9) should expectedly be least during pulmonary venous administration and sequentially higher during pulmonary arterial and airway administration respectively (Fig 6) The model is compatible with all the data in the present work and supports the hypothesis that the region within the lungs sensitive to hypoxia is located extravascularly on the arterial side functionally closer to the airways than to the responding vessels as indicated (×) in Fig 9

At the beginning of the present study L. B was a research fellow of the Norwegian Council for Science and the Humanities He is presently a research fellow of the University of Oslo The work has been supported by grants from Norsk Medisinaldepot and from Institusjonen Hjelpestikkene We thank M Karin Laugerud for skilled technical assistance

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Acta Physiol Scand 1980 109 463–472

A simulation study of a neuron in a simple muscle control system

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LIESTØL, K NJÅ, A. & WALLØE, L. A simulation study of a neuron in a simple muscle control system Acta Physiol Scand 1980, 109 463–472. Received 20 Dec. 1979 ISSN 0001-6772 Institute of Physiology University of Oslo Norway

The mechanism underlying signal transmission through the accessory neuron of the muscle receptor organ in the crayfish abdomen was examined experimentally and by computer simulation. The mathematical model of the accessory neuron and its synaptic input was based in part, on previous neuronal models studied by Stein (1965) and Rall (1964). The model predicted with considerable accuracy the behaviour of the experimental system in a number of situations It was possible to determine in some detail the mechanisms underlying the frequency response characterized by a threshold and an approximately linearly increasing response for stimulus intensities above threshold. Furthermore, we were able to explain the observation that the accessory cell transforms a regular input train from a stretch receptor into an irregular output train. The highly branched structure of the dendritic tree, combined with a firing threshold in the dendrites which prevents action potentials from invading large parts of the dendritic tree seems essential in determining these properties.

Key words Simulation signal transmission, crayfish neuron

The signal transmission through a neuron is determined by the interplay between several of its inherent properties These may include the electrical characteristics of the nerve membrane the geometry of the cell and the function of its synapses with other neurons Since direct examination of these properties is often difficult, it is important to know to what extent the mechanisms underlying signal transmission can be deduced from the relationship between the input and output of the neuron In the present study we have used extracellular stimulation and recording to examine one particular nerve cell the large accessory neuron of the abdominal muscle receptor organ of the crayfish (Alexandrowicz 1951 1967) We started by performing a set of experiments which served as a basis for the construction of a neuronal model. Preliminary analysis showed that a model more complex than those used by previous investigators in similar situations (Stein 1965 Walløe Jansen & Nygaard 1969) was required. The result of computer simulation of the behaviour of this model were then compared with experimental observations.

Our results provide information about the roles of synaptic mechanisms and cell geometry in determining the signal transmitting properties of the accessory neuron

METHODS

Experimental preparation

The dorsal muscles in the thorax and abdomen of several crustaceans possess large muscle receptor organs (Alexandrowicz 1951 1952, 1967). Each of these sense organs consists of a specialized muscle bundle and a peripheral nerve cell body with highly branched dendrites that ramify the muscle. There are only four such receptors in each abdominal segment, two on each side. Each pair consists of a rapidly adapting and a slowly adapting stretch receptor both of which are activated by abdominal flexion (Wiersma, Furshpan & Florey 1953) The stretch receptor axons run ventrally across the surface of the body muscles and enter the abdominal ganglion of the next anterior segment There are also 3 different axons making inhibitory synapses on the slowly adapting stretch receptor neuron (Alexandrowicz 1967 Jansen et al. 1971). The most powerful of these inhibitory axons is that of the large accessory cell, which is the object of this study The slowly adapting receptor causes powerful reflex activation of the large

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The signal transmission through a neuron is determined by the interplay between several of its inherent properties. These may include the electrical characteristics of the nerve membrane, the geometry of the cell and the function of its synapses with other neurons. Since direct examination of these properties is often difficult, it is important to know to what extent the mechanisms underlying signal transmission can be deduced from the relationship between the input and output of the neuron. In the present study we have used extracellular stimulation and recording to examine one particular nerve cell, the large accessory neuron of the abdominal muscle receptor organ of the crayfish (Alexandrowicz 1951, 1967). We started by performing a set of experiments which served as basis for the construction of a neuronal model. Preliminary analysis showed that a model more complex than those used by previous investigators in similar situations (Stein 1965, Walløe, Jansen & Nygaard 1969) was required. The result of computer simulations of the behaviour of this model were then compared with experimental observations. Our results provide information about the roles of synaptic mechanisms and cell geometry in determining the signal transmitting properties of the accessory neuron.

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accessory neuron in the same and neighbouring segments (Eckert 1961). The accessory cells have been particularly useful in studies of the mechanism of synaptic inhibition (Kuffler & Eyzaguirre 1955), inhibition in small nerve networks, i.e. investigations on principles of summation of excitatory and inhibitory impulses (Fenstad et al. 1976), lateral inhibition (Liestøl et al. 1976) and problems of phase locking (Perkel et al. 1964). In the present experiments we examined the correlation between the activity in an accessory cell and the activity in the stretch receptor that provided the reflex input. The experiments were performed on the isolated abdomen as previously described (Jansen, Njå & Walløe 1970, Njå & Walløe 1975). In short, the superficial extensor muscles of each of the abdominal segments were exposed and a suction electrode placed on the muscle nerve for en passant recording of action potentials. The slowly adapting stretch receptor was either activated by flexion of the joint, by stretching the receptor muscle with a hook, or the nerve was cut and its central end stimulated electrically. The preparation was kept in a bath of cold oxygenated physiological saline (12°C).

Intracellular recordings from abdominal ganglion cells were obtained on isolated preparations consisting of each pair of stretch receptors of the third abdominal segment and their nerves in continuity with part of the ventral nerve cord, including the second and third abdominal ganglia. En passant recording and stimulation were done across a seal on each stretch receptor nerve (second root). The ganglia were pinned out ventral side up and decapsulated, and conventional glass microelectrodes filled with 4 M potassium acetate (resistance 20–50 MΩ) were advanced through the cell body layer and into the neuropile.

The model

Several mathematical models have been developed for the analyses of nerve mechanisms or for the evaluation of properties of specific neurons (Rall 1964, Stein 1965, Walløe et al. 1969, see also references in Holden 1976). However, certain properties of the accessory cells (to be described later) made it necessary to combine and extend these previous models.

Our model is defined by 6 assumptions. We will describe the model by stating these assumptions and by giving a motivation for each.

The model cell is divided into compartments, each representing a part of the neuron. Each compartment is regarded as an electrical unit, while both membrane properties and membrane potential may differ between compartments. The compartments are combined into a neuron by the assumption that current may pass between the compartments in a specified way.

Each compartment is based on a model originally formulated by Fatt & Katz (1953). All synaptic inputs are assumed to be excitatory (the model may easily be extended to include inhibitory input). The model may then be illustrated by means of an analogous electrical network. The model assumes that current may pass the membrane along three parallel channels. One channel represents the effect of the membrane capacitance (C). Each of the two other channels consists of an electromotive force in series with a resistance. These two channels represent the effect of diffusion over the membrane under resting conditions and during excitatory influence. G_r ("resting conductance") is constant, while G_e is determined by the synaptic activity (to be described later). With no synaptic input ($G_e = 0$), the membrane potential (V_m) will be equal to the resting membrane potential (E_r). Synaptic activity will cause the membrane potential to approach the reversing potential for the excitatory synapse (E_e).

A mathematical formulation of these assumptions gives the differential equation

$$\dot{V}_m = \frac{G_r}{C}(E_r - V_m) + \frac{G_e}{C}(E_e - V_m)$$

Following Rall (1964), we will introduce a new variable defined by

$$V = \frac{E_r - V_m}{E_r - E_e}$$

V may then be regarded as relative depolarisation, that is, V is equal to 0 under resting conditions and approaches 1 under strong excitatory input. The differential equation then takes the form

$$\dot{V} = -\frac{G_r}{C}V + \frac{G_e}{C}(1.0 - V)$$

Let g_{ij} be the conductance between the i'th and j'th compartment. We will then assume that the current between compartment i and j is equal to $g_{ij}(V_i - V_j)$. Furthermore, we will assume that the change in membrane potential induced by this current is proportional to the capacitance in the compartments. The conductance between compartments i and j will then result in a change in the membrane potential given by

$$\dot{V}_i = \frac{k_i}{C} g_{ij}(V_j - V_i)$$

where k_i are constant.

The neurons in the abdominal ganglia of the crayfish have long, highly branched dendrites (Wine & Hagiwara 1977). However, to obtain a practicable number of parameters in the model, we had to use a simple geometrical structure. Some exploratory trials showed that a two-branch structure was necessary to obtain satisfactory results when simulating situations with two inputs, e.g. controlled and spontaneous. We therefore selected the 16-compartment structure shown in Fig. 1. In most simulations all segments were supposed to have equal membrane properties (with some exceptions, see below), i.e. all C, G_{ri} and k_i were equal for i = 1, 2, ..., 16. This relatively simple choice of structure reduced the opportunities to study some properties of the accessory cells, especially summation of several inputs. However, several of the more important properties show little sensitivity to small changes in structure.

We may now state our first assumption:

(A1) The model cell is divided into compartments as shown in Fig. 1. Within each compartment the electrical properties are uniform. If generation of action potentials is suppressed, the variations in the membrane potential may

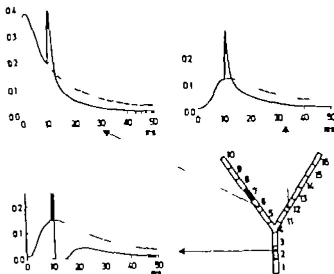


Fig 1 Membrane potential as function of time in three different segments of the models as result of synaptic input in the black segment. Arbitrary vertical its. Horizontal axis time in mm Dotted line firing threshold Broken lines show the response if the cell is not fired by the ynaptic input. The fully draw lines show the response when the cell fires.

be described by the following system of differential equations

$$V \quad \frac{G_i}{C}V \quad \frac{G_j}{C}(1\ 0-V)+\frac{k}{C}\sum_{j=1}^{16} g_{ij}(V \quad V_j) \ =1 \ \ldots \qquad 16$$

here

$$g_{ij}=\begin{cases} 1 \text{ if compartment and } j \text{ are connected} \\ \text{otherwise} \end{cases}$$

and the other parameters have been defined above

The generation of action potentials is described by the following two assumptions

(A..) All impulses are generated in particular segment hich denoted the impulse generation area (segment 2 in Fig 1)

(A3) An impulse is generated when the membrane potential exceed time-independent firing threshold

When an action potential is generated, it will normally propagate both along the axon, and backwards towards the dendrites It is generally assumed that the firing threshold of the membrane in most neurons increases towards the distal parts of the dendrites If this threshold is sufficiently high, the effect of excitation in nearby segments of the cell is not strong enough to propagate the action potential Accordingly in areas with sufficiently high thresholds the influence from the firing of the cell is restricted to passiv conductance In the model cell this is described in the following ay

(A4) The cell is divided in active and passive compartments The active compartment fire simultaneously th the impulse generating area, while the passive part of the cell is influenced solely through passive conductance

In a period surrounding the firing, the membrane potential in the active parts of neuron will be determined by the high permeabilities for N and K The membrane potential will accordingly be independent of influences from other parts of the cell. The exact form of the time variations in the membrane potential in this period is unknown. Different forms of these time variations were therefore tested. A simple description with no after hyperpolarization phase proved sufficient for most situations

(A5) After the generation of an impulse in the neuron, the relative depolarisation (V in assumption 1) in the active segments is equal to 0.5 for 1 ms. The membrane potential is afterwards equal to the resting membrane potential for parameter-specified period

A 5 implies that in the period following firing, the membrane potential is kept at sub-threshold level i.e the cell is refractory The effect of introducing additional refractoriky absolute or relative by assuming a high threshold in period after firing is very small for moderately long refractory periods No such period were therefore introduced.

The effect of ynaptic activity in chemical ynapse is to change the conductance for one or more ion species. In our model this correspond to changes in G_s in assumption A1 More precisely we will assume

(A6) The change in conductance in one compartment caused by an impulse reaching synapse in that compartment at time t_0, is given by

$$G_s(t)=G_{max}\ \exp(-\mu(t-t_0)) \quad t>t_0$$

G_{max} and μ are parameters that may vary between ynapses. The conductance changes from several impulses are summed linearly

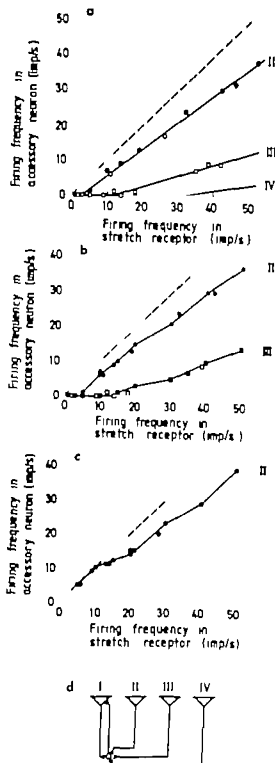


Fig 2 (*a*) Firing frequency in an accessory neuron as a function of the stretch receptor input Open circles input from one of the two nearest neighbours (II in part *d* of the figure) Open squares input from the second nearest neighbour (III in *d*) The straight lines indicate generalization from this and other expts (*b*) The firing frequency in an accessory neuron as a function of the stretch receptor input Circles input from the nearest neighbour Squares input from the second nearest neighbour Open symbols empirical results. Filled symbols best fitted model (*c*) As Fig 2*b* but from the other side of the same animal The simulation re ults in *b* and were obtained with identical parameter values in the model except that the level of pontaneous input was increased from *b* to *c* (*d*) Schematic drawing of the neuron network

The time course of the conductance changes is to some extent arbitrarily chosen The as umption corresponds to the expected time course if the conductance is assumed to be proportional to the number of activated transmitter receptors If a certain number of receptors are activated immediately when an impulse reache the synapse if m receptors are activated later and if the probability of deactivation of an active receptor is constant Except when μ is small (which correspond to long duration of the conductance change) small changes in the time course have negligible effects on the behaviour of the model neuron

Stochastic elements in the transm tter releasing process was introduced in one version of the model by setting $G_{max} = k \cdot N$ where k is a constant and N is a Poisson or binomial random variable representing the number of released synaptic vesicles. Time may be introduced in the release process by describing it as a time dependent Poisson process

Facilitation wa introduced by making G_{max} dependent on the time interval since preceeding impulses An approximation to results obtained by Mallart & Martin (1967) and Magleby (1973) was achieved by making G_{max} proportional to $A_o + A \cdot \sum e^{-\delta \Delta t}$ where A_o, A and δ are parameters and Δt is the time interval since preceeding impulses

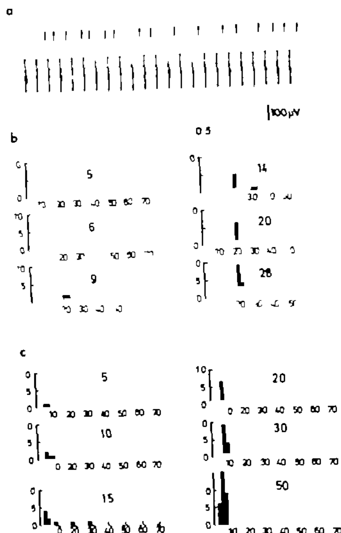
A computer version of the model wa written in the programming language SIMULA 67 and run on the CDC Cyber 74 computer at the University of Oslo Different experimental situations could then be simulated by activating synapses in a time pattern corresponding to the expected synaptic activity in the experiments (including different levels of uncontrolled spontaneous input)

At first impression of the behaviour of the model neuron may be obtained by looking at Fig. 1 The time course in three compartments of an EPSP caused by an impulse to the 7th compartment (Fig 1) is shown Note e pecially the change in the time course as we move away from the 7th compartment and the effect of a firing including the memory mediated by the passive compartments (compartment 6–10 and 1 16 were pas ive in this simulation)

RESULTS

Experimental results

It was a typical result that the firing frequency of the stretch receptor had to exceed a threshold before the accessory cell started firing When the frequency of the input exceeded this threshold the response increased approximately linearly with the input (Fig 2*a*) When the stimulus derived from more distant receptors the threshold was greater and the increase above threshold was smaller In



F g 3 ( ) Refle activation of an accessory cell The lower tracing show spikes in the receptor axon the upper tracing shows the resulting spikes in the accessory cell In this xperiment the slowly and rapidly adapting receptors were co-activated at constant rate by pulse-fed electromagnetic tretcher (*b*) Distributions of latencies in one accessory cell hen it is activated by the stretch recepto in one of its neighbouring segments Horizontal axis in each of the 6 panels is time in ms Vertical xis number of spikes with the corresponding latency Numbers above each distribution show the mean firing frequency in the stretch receptor which is activating the accessory cell. ( ) Distributions of latencies in simulation expts on the model Axes as in *b* Note that the distributions how in *b* include the conduction time in the stretch receptor axon and in the axon of the accessory cell, while does not

Fig -a the results for receptors numbers II and III are from the same animal while the line for receptor IV is based on experience from other experiments

One implication of these observations is especially important. Since threshold exists, one EPSP generated by the input from a single stretch receptor cannot alone exceed the firing threshold of the accessory cell However this threshold was consistently seen in preparations where the accessory cell might fire at each of 2–3 succeeding input impulses at high stimulus intensities (mechanical or electrical) of the stretch receptor This behaviour is

difficult to explain without assuming that the cell after a firing still contains information about events before the firing. This motivated the introduction of some sort of memory in the model either in the synapses as facilitation or longlasting transmittor effect or in the membrane by assuming that the firing of an action potential only resets the membrane potential to resting levels in part of the neuron (assumption 4 in the model description).

Another characteristic of the signal transmission through the accessory cells is shown in Fig. 3. A firing in the accessory neuron may usually be ascribed to one specific input impulse (Fig. 3a). Fig. 3b shows the results of measurements of the time between action potentials in the receptor axon and in the axon of the accessory cell. If the approximately constant (but unknown) conduction times are subtracted, the measured values represent the time taken from the synapse of the accessory cell receives an impulse until an action potential is generated. These values will be called transmission times. Two observations on transmission times are essential. First, there are marked stochastic variations in the transmission times. Second, the 'expected' transmission time varies as a function of stimulus intensity, with short transmission times connected to high stimulus intensities.

Some sort of systematic variation between different preparations exists. This is illustrated by the difference between the two sides of the animal shown in Fig. 2b and c. The variation is typically restricted to low stimulus intensities. In some preparations the thresholds in the frequency curves are low (or even absent) and the stochastic variation in the transmission time high. With increasing thresholds, the variation in transmission time diminishes.

Simulation results

Fig. 2 shows how the model neuron after adjustment of the parameters reproduced the experimental results on the frequency response of the accessory cell. The fit to the data for stimulation of receptors II and III in Fig. 2b were obtained with equal parameters except for G_{max} in A6, which determines the strength of the synaptic input. Fig. 2b compared with 2 also show how the variation between preparations may be explained by differences in the level of activity in neurons other than the stretch receptors. By increasing the number of spontaneous impulses in the model, the response of the model neuron changes from a pattern close to the experimental results from the left side of the animal (2b) to a pattern close to the results from the right side (2c). Both in accessory cells and the model neuron a high frequency threshold was associated with a low level of random variations in the transmission times, while the stochastic elements were marked when the firing threshold was low.

Fig. 3 shows how the transmission time variations were reproduced. Together with Fig. 2 this figure demonstrates that a close agreement between empirical and simulated results may be obtained. However, since the model includes a large number of parameters, good agreement might have been obtained even if the model cell was far from analogous to the accessory cells. A primary examination of whether the close agreement was a multiparameter artifact was obtained by looking at other aspects of the results from the same experimental situation. The length of the transmission time was for instance in a systematic way dependent on whether or not the preceeding impulse from the stretch receptor had fired the accessory cell. The model neuron behaved in the same way, and only small additional adjustments of the parameters were necessary to obtain a good agreement (Fig. 4).

Signal transmission mechanisms in the model neuron

The firing pattern of a neuron is determined by the variation in the membrane potential in the impulse generating area. The effect of a certain nerve mechanism is accordingly mediated through its effect on the membrane potential in the impulse generating area. In the following, we will therefore first describe characteristics of the synaptic potentials in the impulse generating area, and then look at the mechanisms responsible for these characteristics.

The synaptic potentials had to be of long duration, at least 40–50 ms, from the start until the depolarization was insignificant. Furthermore the rise-time (the time from the start of the depolarisation to the maximal depolarisation) had to be long, approximately 10 ms. The amplitude of the potential varied in simulations of different preparations, but for impulses from stretch receptors in the nearest neighbouring segments, the amplitude seemed to be approximately 0.8–0.9 times the difference between the firing threshold and the resting membrane potential. The rise-time could be deter-

...ured relatively accurately while the total duration to a larger degree was dependent on other parameters

The long duration of the synaptic potentials is in the model cell caused by a small resting conductance relative to membrane capacitance (i.e. G_r/C is small in A1). The long rise-time is caused by a considerable electrical distance between the synapses and the impulse generating area (see Fig. 1 and for more thorough discussion of distance effects, Rall 1964)

Memory mediated by depolarisation in the passive compartments gave thresholds combined with a stable linear form of the frequency curve for higher stimulus intensities. A relatively large part of the neuron (measured in electric capacitance) had to be passive. The best fit to the experimental data was obtained when approximately half of the compartments were passive

To understand the variations in transmission times, one has to look at the combined effect of the spontaneous impulse activity and the long duration of the synaptic potentials. The slow EPSP's from the stretch receptors bring the membrane potentials close to the firing threshold for a long period. A extra impulse in this time interval will then fire the cell. In this way we obtain a system where an impulse from a stretch receptor causes a high probability for firing, but where the exact moment for firing is often determined by the additional synaptic input. Accordingly we obtain a randomisation of the firings

The simulations indicated that the spontaneous impulse activity was fairly high in most experimental situations. Some of the impulses had to generate relatively large synaptic potentials with amplitudes of at least 1/3 of the firing threshold in the impulse generating area. The time pattern of the spontaneous activity was not important as long as the impulse trains were somewhat irregular and not strongly correlated with the activity in the stretch receptors

The long rise-time of the synaptic potentials creates an additional stochastic component in the transmission times. If the membrane potential is high before the new impulse from the stretch receptor arrives, only a short time will elapse before the new EPSP has brought the membrane potential above the firing threshold. A low initial membrane potential will cause a correspondingly longer transmission time. If for instance no action potential is generated after an impulse from a stretch receptor the membrane potential will remain high when the next impulse arrives. Accordingly the transmission time will be short as shown in Fig. 4

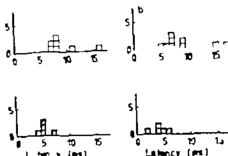


Fig. 4. Distribution of latencies as obtained from an accessory cell ( ) and from model simulation (b). Hatched squares: The cell was not fired by the preceding EPSP. Open squares: The cell was fired by the preceding EPSP

Intracellular recording from neurones in abdominal ganglia

We have now shown that the model can reproduce certain aspects of the results in one experimental situation. In order to explore the similarity between the model and the experimental system further we tried to obtain intracellular recordings from the large accessory neurone. Its cell body has been located to the center of the ganglion lying posterior to the one from which the axon emerges (Eckert 1961, Wine & Hagiwara 1977). No neuronal somata impaled in this region showed any synaptic potentials. However processes impaled in the neuropile frequently showed spontaneous excitatory and inhibitory synaptic potentials of several mV amplitudes (see also Calabrese & Kennedy 1974)

The resting membrane potentials in these processes were between 40 and 70 mV but overshooting action potentials were not seen except in a few recordings which appeared to be from axons. Although processes which showed excitatory synaptic potentials correlated with stretch receptor firing were impaled in a large number of preparations spike activity characteristic of the accessory cell was only seen once (Fig. 5). Unfortunately the neurone impaled in this case could not be identified by testing its inhibitory action on the stretch receptor firing (Eckert 1961, Jansen et al. 1970) since conduction in the accessory cell axon blocked accidentally in the second root in this preparation

Several of the neurones impaled showed parts of

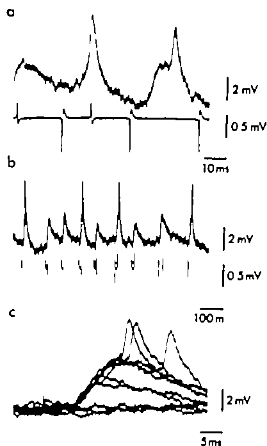


Fig. 5. Intracellular recording from a neuronal process in the neuropile. (*a, b*) Simultaneous recording of the membrane potential in the neuronal process (upper trace) and the spike activity in the ipsilateral intact second root of the next anterior ganglion (lower trace) during stretch activation of the slowly adapting receptor (large spikes). The small spikes in the nerve record shows a reflexly activated motoneurone. (The spike of the large accessory cell, while initially present, was lost prior to this recording together with the reflex inhibition of the receptor, presumably because of nerve injury at the site of extracellular recording.) (*c*) Electrical stimulation of the second root. The short-latency subthreshold EPSP was produced by the rapidly adapting receptor and the second EPSP by the slowly adapting one, as confirmed by natural activation. Strongly attenuated action potentials occurred with variable latencies. The resting membrane potential was close to −50 mV. Several sweeps were superimposed at different stimulus intensities.

the behaviour which according to the simulations should characterize the accessory cells, e.g. long lasting synaptic potentials or passive membrane. In the impalement shown in Fig. 5 the overall behaviour was very close to that predicted for branches of the accessory cell.

— The synaptic potentials had a very long time course which approximated the form in the model cell (compare Fig. 5 with Fig. 1).

— The membrane potential in the recording area was not reset by the action potentials, i.e. this region of the membrane was passive.

— EPSP's with significant amplitudes from spontaneously active neurons were recorded.

Accordingly, the intracellular recordings showed that there are ganglion cells innervated by the stretch receptor with the properties predicted in the simulations. The usefulness of the accessory cells for more detailed studies on neuronal mechanisms is, however, seriously restricted by the difficulties of obtaining intracellular recordings.

DISCUSSION

Rall (1964) has shown, through theoretical considerations, that the form of a synaptic potential may undergo significant changes during the spread through the dendritic area. The present investigation on the accessory cells showed that, in this neuron, the geometry has marked effects on the transmission properties of the cell. The effects were mediated by at least two mechanisms. First, the changes of the form of the synaptic potential during the spread from the synaptic area include a much prolonged rise time. This contributes to the stochastic variation in the times of firing of action potentials. Furthermore, we have shown that the cell may be divided into two parts: in one area the membrane is active, in the other it is passive. As described above, the passive membrane represents a "memory" of earlier impulse activity which is essential for the frequency response of the neuron. The passive membrane also seems to have a linearing effect on summation of several inputs. Accordingly, the function of the accessory cells is a good illustration of how the geometry of a neuron affects its function.

Long lasting effect of the transmittor would increase the duration of the synaptic potential, and might even represent a form of memory after a firing. The simulations could separate the effect of this form of memory from other possible mechanisms, and long-lasting transmittor-effect could be ruled out as the essential memory mechanism in the accessory cells. However, it was not possible to determine whether a long-lasting transmittor-effect was part of the explanation of the long duration of synaptic potentials.

As response to a regularly spaced input from a stretch receptor, the accessory cell will produce an

irregular train of impulses According to the simulation results most of this irregularity is caused by the combined effect of long-lasting EPSP's from the stretch receptor and spontaneous input from other neurons. If the number of synaptic vesicles released per impulse is small this might provide an alternative source of stochastic variation It was however not necessary to add this mechanism to explain the experimental results and it was not a probable mechanism for explaining the long transmission times found in most preparations at low stimulus intensities

Small changes in the properties of the accessory cells could reduce its randomising effect but it rather looks as if the cells are constructed for maximizing the randomisation (of course under some constraint) This may seem unfortunate since one normally expect that regularly spaced input would have the highest information carrying capacity However a closer look at the system reveals that a regularly spaced input from the accessory cells may cause phase-locking in the stretch receptor (Perkel et al 1964 Løestøl, in prep) The peculiar effect of this is that for certain frequency shifts in the accessory cell the high frequency of inhibitory impulses may have a lower inhibitory effect than the low frequency A way to avoid such phase locking is randomisation. The randomisation mechanisms in the accessory cells may therefore represent suitable adjustment for avoiding destructive phase-locking

Facilitation represents an alternative explanation of the memory of earlier synaptic activity after a firing. Since several different mechanisms may produce facilitation statements about the importance of facilitation in the accessory cells must be made with caution By introducing facilitation according to the description given by Mallart & Martin (1967) and Magleby (1973) in our model frequency thresholds could easily be obtained However for stimulus intensities above threshold facilitation caused the frequency curve to bend sharply upwards creating clearly non-linear curve Even combined with synaptic depression it seems unlikely that facilitation would create stable linear frequency curve like the one observed in our experiment In contrast memory caused by depolarisation in the passive compartments gave thresholds combined with remarkably stable linear form of the frequency curve for higher stimulus intensities. Facilitation was also studied in set of experiment where the accessory cells were stimulated alternatingly by pairs of impulses from one and two stretch receptors. The results supported the conclusion that no significant facilitation was present in these synapses (in contrast to the pronounced facilitation found in many neuromuscular synapses in the crustacea, see i e Frank 1973)

The applicability of simulation methods will depend on several factors Here we shall only discuss the importance of the amplitude and time-course of the synaptic potentials If we compare the results of our investigation with the results of other similar studies such as the examination of DSCT-neurons in cat by Walløe et al (1969) some marked differences appear In order to describe these differences we must first give a brief summary of some result on the summation properties of the accessory cells and the DSCT-neutrons. Examination of the summation properties of the accessory cells was conducted by stimulating the cells with sequences of pairs of impulses alternatingly from one or two stretch receptors In our context the interesting result is that the simulation studies showed that the response of the cell depended significantly on the geometric structure of the neuron and on the relative position of the synapses. Accordingly the structure is expressed' in the cell's response and inference about this structure may be made from the electrophysiological results On the other hand it i difficult a priori to predict the summation properties of the accessory cells. In some sense the DSCT-neuron represents the converse situation. In DSCT-neurons the synaptic potential s smaller 3–4 impulses are needed simultaneously to fire the cell The exact form of the synaptic potentials will then be of less importance due to the summation of several potentials and the output signal will contain little information about the geometrical structure of the neuron In the studies of Walløe et al the problem was to reveal unknown properties of the input to the neuron. A relatively simple one-compartment model was used with success Amongst other things it wa possible to determine the number of muscle spindle afferents converging on each DSCT neuron and the importance of this number to the transmitting properties of the cell I general neuron with large synaptic potentials such as the accessory cells or for example cells n mammalian sympathetic ganglia, will represent a problem in situations where description of summation properties i needed but an advantage if the

purpose is to examine other inherent mechanisms of the neuron

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COMMUNICATIONS

4 A

C 1

Influence of the Na K-activat d ATP ase on resis tance ve sel r ctivity in normotensive rats compared with t model f p imary and secondary hyp rten ion

G GÖTHBERG B JANDHYALA & B FOLKOW

Dep rtment f Physi l gy Uni ity f Göt borg Sweden

T inve tig t the rol f N -K-activat d ATP-ase in the regulati f re i tan v sel re c-tivity pair-perf sed hindquart t bmique wa sed i Wi ta Kyot (WKR) dinary Wistar normo tensive cont l (NCR) pontaneously hyperten ive (SHR) and two kidn y-on clip ren l hyperten ive rat (RHR) Ouabai was utilized t inhibit the sodium pump in v scul r smooth muscl

Ouabain up t a p f sat on ent tion f 10^{-4} did not alone noticably nhanc va cular i tanc in the present ituation f rel tively low constant flow Howeve vasocon t i t r f f ct f no ad enali (NA) aopre in (VP) and ba ium hl ide (Ba^{++}) were onsi tently enhanc d whe ouabain wa dd d xcept when maximal go-ni t ffect had lready been as hed ED_{50} val es bef re and ft ouabain addition were computed from the log do re i tan respon curve f v rious agoni t and th hift ca ed by ouabai i ref rred t as ouabain tivity index (OAI) omput d f llows

OAI ED_{50} f goni t/ED_{50} f goni t ouabain

The we no ignificant OAI diff enc *between NCR and WKR In SHR howeve OAI we f* 11 thr goni t ignificantly great than in normoten i t i dicating an incre sed ti i ty f the ouabai en iti sodium pump i smooth mu cle f the SHR istanc vessels The ituation i RHR was t lea t in pa t diff rent ine OAI f g BA^{++} did not diff r significantly between RHR and normotensive controls

Based on these finding the f llowing conclusion may be rea hed

1 The responsiven f re i tance vessel t constri t agent i t considerable degree in fluen ed by the activity lev l f the Na-K acti t d ATP se

2 In SHR re i tance ve sel the sodium pump acti vity a ems t be in ea ed perhap t compensat f r an alt red pa ive sodium p rmeability The OAI lt ations in RHR representi g secondary hy-perten ion were l onsi tent f the agonist used and may refl t a smooth muscl lt ration diff rent from that in SHR p imary hyperten ion

3 *The sugge tions f an inc eased sodium pump ac*-tivity in SHR vascul smooth muscl i in keeping with b ervation by ther inve tig tor on ryth-rocyte from SHR and senti l hyperten ive pati ent indi ting an in reased pa ive sodium per-meability that i partly ffset by accentuated pump ctivity

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C 2

Vibrations cause electro-mechanical uncoupling in the spontaneously active longitudinal muscle of the rat portal vein

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In previous studies from this laboratory vibrations have been found to reduce active force development in isolated vascular smooth muscle There is indirect evidence that this inhibition is caused by a direct action on the contractile process (Ljung & Sivertsson 1975 1980)

In order to elucidate the effects of vibrations on the electrical membrane discharge and on the contractile force the spontaneously active longitudinal smooth muscle of the isolated rat portal vein was studied The vein was mounted vertically in an organ bath filled with modified Krebs solution between a submerged isometric force transducer and a vertical rod extending from a servo-controlled vibrator The electrical activity was recorded extracellularly The rectified signal was integrated over 1 min periods and related to the mean isometr c active force developed over the same time pe iod

Sinusoidal vibrations (40 Hz 2 5-30 % tissue length peak to peak) applied in the longitudinal direction of the smooth muscle caused prompt and reversible reduction of the active force In contrast neither the pattern of the phasic contractions nor the membrane discharge were altered The amplitude of the active force decreased with vibration amplitude being 60 % of control at a vibration amplitude of 12 ± 4 % (mean ± SD n 8)

Oscillations with characteristics corresponding to those of the vibrations applied in the present experiments may prevail in peripheral blood vessels in various occupational situations as well as under pathophysiological and possibly physiological circumstances

It is concluded that vibrations of vascular smooth muscle cause dissociation of electrical membrane discharge and mechanical force development This finding of electrical-mechanical uncoupling provides further support to the previously forwarded hypothesis that vibrations cause inhibition of smooth muscle contraction by a direct action on the contractile mechanism It is likely that the induced length changes lead to increased rate of detachment of the acto-myosin cross-bridges

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C 3

Influence of initi l microvascul resistance on the magnitude f a superimposed myogeni microv cular constrictor response; importance f physical factors

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Dept. of Phys logy and Biophysics University of Lund Sweden

It has been suggested g by Myers and Honig (1969) that the magnitude of response i the resistance vessel to a g ven vasomotor t mul is increased with increa ng level of vascula tone prese l ng prior to the stimulus Thi f n-ding i l ne with previous study from our laboratory (Grände 1979) demonstrat ng that the magnitude of dynamic myogenic constrictor re-sponse to standardi ed transmural pressure sti mulation gn ficantly and gradually ncreased with increa ng level f icrovascula tone

The present study as ttempt to est mate the importance of poss bl phys cal factors beh nd the reinforc ng phenomenon by making correspon-d ng analys s using ou prev ously publ shed ma-thematical model for the microv scula bed The model was based on force-equ librium the crvessel wall includ ng pas ve forces rel ted to vascular transmural pressure l st city wall viscosity and act ve myogenic forces ssumed re-lated to wall tens on and ts rate of change (for deta l of the model see Borgstrom & Grande 1979)

This analysi demonstrate great resemblance between the dynamic icrov scul res stance re-sponses obta ned with the model and correspond ng curves observed i vo and nd cates the rei forcing phenomenon to be mai ly ascribed to an i crease i gai implicit the inverse fourth power relationship between microvascula resistance and internal vessel radius according to Poiseuille's law though countera ted by ffects of Laplac law and to some extent by concomitant effects of shift long the smooth muscle length-tension curve

It can be concluded that physical factors sig-ificantly must add to the complexity in overall vascul control and that the radius factor i Poiseuill s law per se nevitably leads to vast devi tion from l nearity i circulatory resistanc regul tion The phenomenon could also bear some rel t onship with the ncreased reacti ity in hypertensive compared to normotensive rats which accord ng to the Folkow hypothes mainly can be expl ined by tructural increase of wall lumen ratio (g F lkow et al 1970)

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C 4

Plasma colloid osmoti pressure i venous blood from the foot f man i the sitting pos tion

R. [illegible] [illegible]LAND [illegible]LAND

Institute f Physiology University of Oslo and Institute of Physiology Univers ty of Bergen, Norway

slow te f edema formation in the legs human standing or sitting quietly has ttracted the interest of physiologists for many years Increased interstitial hydrostatic pressure and decreased area availabl for fi tration are among the explanations of ered Hellander Obery Öhlrem (1964) deduced from observations of the filtration coeff cient tha the filtration area was greatly educed due to closure f precapillary sphincters following change from the supine to the erect position. The capillaries would however be expected to remain open when the venous pressur is high even if precapillary sphincters are used Ear data from Youmans et al 934 suggest tha venous plasma osmoti pressur the leg-down position.We wished to llow up and expand this pioneer study A low flow through the vascular bed coul cause the evaluation of iquif cant increase in lasma colloid osmoti pressure and ow te f fi tration because the increased colloid osmotic pressur coul parti counter-balance the in-creased capil ary pressur An increased plasma c-l oid osmoti pressure would cause an error in th alcula ion the ltration coef icient because thi coe f ent is usual y calculated on th bas the the net ltration pressur increases in proportion to the venous pressure In the seated relaxed human comfortabl environmental temperature with the foot being about 60 cm below heart level we found the colloid osmotic pressure in plasma from blood sampled from vein on the dorsum f the foo to be about 5 mm Hg higher than in plasma from the arm This measurement probably grossly underestimated the increase in capillary colloid osmoti pressure due to admixtur f blood flowing through arterio-venous anastomoses We repeated the experiment with the subject in cold environment and now the measured coll id osmoti pressure was about 15 mm higher in blood from the foot than from the arm. We do not know however to wha extent admixture f blood flowing through arterio-venous anastomoses still took place

We conclude tha rise in capillary colloid osmoti pressur may significantly contribute to prevent edema i the legs of the erect immobil human

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C 1

Influence of the Na-K-a tiv t d ATP ase on resis tance vessel reactivity in normoten ive r ts compared with r t model f primary and secondary hyperten ion.

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Dep rtme t f Phy i logy University f Göt bo g Sweden

T inve tig t the rol f Na-K-activat d ATP-ase in the regul tion f istanc ve sel re tivity p ir-perfused hi dquart r technique wa sed i Wi t Kyot (WKR) rdinary Wi ta normotensive cont 1 (NCR) spontaneously hyperten ive (SHR) and two kidn y-one clip ren l hyperten ive rat (RHR) Ouabain wa tilized t inhibit th sodium pump in vascul smooth muscl

Ouabain up to perfusat con entration f 10^{-4} did not loos noticably enhanc vascular i tanc i the pr ent situation f l tively low constant flow Howeve v ocon t i t ef f t f radrenalin (NA) sopre in (VP) and ba ium hl ride (Ba^{++}) were consi tently enhan d when ouabain was dded ex ept when maximal go- i t ffects had l eadly been reached ED_{50} val bef and afte ouabai addition were comput d from the l g dose-r istan re pon curv f v ious gonist and the hift ca d by oua bai i f rred t ouabain ctivity index (OAI) comput d f llows

OAI ED_{50} f goni t/ED_{50} f goni t ouabain

The we no ignifi ant OAI diff en a between NCR and WKR In SHR howeve OAI we f ll thre goni t ignificantly great than in normoten ive r t indicating an incre ed ti i ty f the ou bain sen iti sodium pump i smooth muscl of the SHR resi tanc vess l The ituation i RHR was t least in pa t diff rent ince OAI fo g Ba^{++} did not diffe ignificantly between RHR and normotensive control

Bas d on these fi ding the f llowing conclusion may be re ched:

1 The respon ivenes f re istanc vessel t onst i t r agent i t a conside abl degree in fluenc d by the activity level f the Na-K-acti vated ATP-ase

2 In SHR resistance ve sel the sodium pump act vity seems t be in eased perhap t compensat for an It red pas ive odium permeability The OAI lt tion in RHR representing secondary hy perten ion wer l consi tent f the agoni ts used and may r flect a smooth mus l It ation diff ent from that in SHR p imary hypertension

3 The sugg tion f an in reased sodium pump a ti ity in SHR cular smooth muscl is in keepin with ob rvations by oth inve tigat on ryth cyt s from SHR and essenti l hyperten ive p ti ent indicating an increased pas ive sodium permeability that i partly ffset by ccentuated pump ti ity

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C 2

Vibrations cause electro-mechanical uncoupling in the spontaneously active longitudinal muscle of the rat portal vein

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In previous studies from this laboratory vibrations have been found to reduce active force development in isolated vascular smooth muscle There is indirect evidence that this inhibition is caused by a direct action on the contractile process (Ljung & Sivertsson 1975 1980)

In order to elucidate the effects of vibrations on the electrical membrane discharge and on the contractile force the spontaneously active longitudinal smooth muscle of the isolated rat portal vein was studied The vein was mounted vertically in an organ bath filled with modified Krebs solution between a submerged isometric force transducer and a vert cal rod extending from a servo-controlled vibrator The electrical activity was recorded extracellularly The rectified signal was integrated over 1 min periods and related to the mean isometric active force developed over the same time period Sinusoidal vibrations (40 Hz 2 5 30 % tissue length peak to peak) applied in the longitudinal direction of the smooth muscle ca sed prompt and reversible reduction of the active force In contrast neither the pattern of the phasic contractions nor the membrane discharge were altered The amplitude of the active force decreased with vibration amplitude being 60 % of control at a vibration amplitude of 12 ± 4 % (mean ± SD n 8)

Oscillations with characteristics corresponding to those of the vibrations applied in the present experiments may prevail in peripheral blood vessels in various occupational situations as well as under pathophysiological and possibly physiological circumstances

It is concluded that vibrations of vascular smooth muscle cause dissociation of electrical membrane discharge and mechanical force development This finding of electrical-mechanical uncoupling provides further support to the previously forwarded hypothesis that vibrations cause inhibition of smooth muscle contraction by a direct action on the contractile mechanism It is likely that the induced length changes lead to increased rate of detachment of the acto-myosin cross bridges

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C 7

The effect of own ether on the ionic cu rent g the myelinated nerve memb ane

P ARHEM & H. KRISTBJARNARSON

Nobel Institut for Neurophys ology Karolinska Institutet, Stockholm, Swed n.

The properties of ionopho ha mainly been studied on rtificial memb ane (Haydon 1970) Very little I known of thei affects on natural excitable memb ane Our aim with the p ent study was to analy the ffects on uch natural membrane namely the membran at the node of Ranvier

V studied the ffects on the iom currents in fibres from the clawed toad Xenopus laevis by potential clamp method (Dodg & F ankenha us 1958). A membe of ionophore we t sted but only the crown ethe dicyclohexyl 18 crown 6 was found to chang permeability pa ameter

The most triking affect (in the concentration range 1 100 µM) wa an nactivation of th potassium current ociated with pos ti potential step. The potas ium cur ent decr ed exponentially with tim constant of bout 10 ms concentration of 10 µM.

The effe disappeared gradually afte epola isation to the holding potential. The time t reach 50 % covery of epolari tion to 70 mV was about 100 m t concentration of 10 µM. The recovery time did not depend ma kedly on the holding potential.

Th affect depended clearly on ext rnal [K] In t st solutions with high [K⁺] the inactivation wa] pronounced than in lution with low [K] The decr of the outward pota sium cu ent wa proportionally equally larg as the decrea of the inward potassium cur ent oci ted with polarisation aft positiv potential tep. Thus ctifi tion was observed.

Some f the finding may be f elevanc for th knowledg on the pota ium transport yst m in the nerv memb ane It is ften as umed that thi ystem consist of long po with gating mechanism t th axoplasm end (Arm trong 1975) The main conclu ion f om the pr ent tudy i that our ults do ot support such simpl model of the pota ium ystem.

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C 8

Facilitation of insp tion by low threshold lung mechanoreceptors

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Vagally medi ted excita ry eff ct on phreni nerve activity elicited by pid aflations and d flations have been epeatedly described. In ddi tion to the facilitatory ff ct of large increases in flow (1), it ha be re ently sugg sted that volume dependent excitatory eflexe als exist (2, 3). Experiments we performed in 11 anae sthetized cats (pentobarbitone PB) t stabli h and further char cteris the effect of arying volume feedback on inspiratory ctivity in the phrenic (P) and external inter ostal (EI) nerv P and EI tidal olume (V) flow (V̇) tracheal pressure (TP) and P_{ACO_2} we simultaneously recorded. Integrated P and EI we compared with and without aff ent feedback from lung movements in spontaneously b eathing animals (tracheal occlusion) and with varying feedback in paralysed cats ventilated with rve- pl ume' gov rned ither by phreni activity different gains or by phreni trigg ed ramp in flations of varying lope and amplitude The computer rag of 5 t br aths we ompared to the rag of 5 preceding b eaths

In lightly anaesthetized cats, aft varying delay from the on et of inspiration volume chang re lted an excitatory ffect on both P and EI. The change in P EI from control was linea ly elated to change in TP and V but not to V̇ Thus inspiratory facilitation ould be evoked by small volume increments at unchanged value of V̇ The ffect occurred ontinuously in every breath. Small additional dos f PB (4-6 mg/kg) aboli h ed the excitatory effects without impairing the vagal inhibitory effects on inspiratory duration (T_I) no the inspiration facilitating eff cts of pid inflations Although vagotomy eliminated the volume facilitatory ffect, excitatory effect ould be produced in EI by electrical agal stimu lation at imilar threshold strengths equi ed to produc effects on T_I T produce excita ory effects on P larg stimulus strength we qui ed. P and EI really esponded quantitatively diff ntly to the ame volume stimuli in that any given volume chang produced larger lati chang in EI than in P Afte vagotomy EI was often consid ably educed whe ea P wa la ti ly l affected.

These ults sugg t that olume lated in formation f om low thr shold lung mechanor cep tor can ex rt an excitatory effect on inspiratory moto euron output. Howeve this facilitating effect probably doe not involve the R_β neuron in it entral pathway as doe the inhibitory eflex.

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6 A

C 5

Metaboli esponses to graded xercise i ts

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We have recently developed tiny metabolism chamber including treadmill for the study f exerci ing rats and shown that befor graded exercise test it is nece sary to introduce 20 min pre-test period during which unspecific tres responses disappear (Sonne & Galbo 1980)

Th aim f this study was to evaluat the ffect f running speed on blood l tate (L) and glucose (G) concentr tions on pH oxygen (P_{aO_2}) and carbon dioxid (P_{aCO_2}) tension in arteri l blood and on oxygen uptake ($\dot{V}_{O_2}$) heart t (HR) respiratory exchange rati (R) and rectal temperature (T_R) and to evaluat post-ex rcise changes i these parameters

7 rats had a cathet implanted in the l ft commo carotid art ry the tip f the catheter being freely floating in aorta 11 days later they ran t an inclination f 0% and running peed f 10 m min^{-1} for 30 min Thereafter the speed was stepwise increased with 5 m min^{-1} every 10 min Blood was withdrawn in the 30th 40th tc min f exercise and in the 3rd min after exercise

L G V_{O_2} HR R and T_R increased gradually (P 0 05) from 1 12 ± 0 21 mmol l^{-1} (mean ± S E) 6 88 ± 0 13 mmol l^{-1} 4 1 ± 0 2 ml O_2 (STPD) (min 100 g)$^{-1}$ 456 ± 24 beats min^{-1} 0 76 ± 0 03 and 37 6 ± 0 4 °C espectively t running speed f 10 m min^{-1} to 2 62 ± 0 69 7 51 ± 0 71 5 7 ± 0 2 (about 65% of V_{O_2max} for ts) 520 ± 13 0 83 ± 0 01 and 38 1 ± 0 2 respectively t speed of 20 m mi^{-1} the highest speed all ts were abl to cope with whereas P_{aCO_2} decreased from 39 ± 2 mmHg to 31 ± 1 In the 3rd mi after exercise V_{O_2} HR, V_{O_2}/HR and T_R had decreased gai (P < 0 05) compared to the la t values obtained during work R L and P_{aCO_2} had not changed and G had increased further to 8 35 ± 0 69 (P < 0 02) P_{aO_2} pH and hematocrit remained unchanged t 110 ± 3 mmHg 7 42 ± 0 01 and 38 ± 1% respectively throughout the expt.

The marked increase in glucose and the small increase i lactat concentration are compatibl with the fact that ts have relatively larger live glycogen tore and smaller muscl glycogen store than man The increase in blood glucose concentration during the expt indicates that in rats hepati glucose production i not always ccur tely adjusted to peripheral glucose utili zation on ome t to moment basi The decrease in V_{O_2}/HR fter exercise indic t a decreased troke volume and/or a decreased arterio-venous oxygen difference soon after work

The rapid post-exercise changes bring into dis credit many previous studi of exercising ts using post-exercise sampling

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C 6

pO_2 and pCO_2 in huma muscl during exhaustive stati contraction

F Bonde-Petersen & J S Lundsgaard

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The probe described by Lundsgaard t al (1980) for measurements f gas tensions i blood was used The probe consists f 1 mm stainles tube fitted with porous tip f i tered bronz covered with 20 mikron polyethylene membrane The probe was introduced in m vastus laterali in 4 subjects through small incision in skin and f sci The steel tube was connected to the inl t f 4-channel ma spectrometer (Micromass) As the pre sure in the spectrometer is 10^{-5} Torr g se will diffuse from the urrounding muscl tissue through the polyethylene membran The tensions f Ar O_2 CO_2 and N_2 were recorded on 4 channel strip chart recorder The probe was alibrated in vitr t 37°C in isotoni saline equilibr ted with tmospheri ai and known CO_2 tension during tirring The low solubility for O_2 in tissue (0 021 ml/ml/Atm) creat an increa sed diffusion resistance which ttenuates the pO_2 signal from th muscl However in vitro periments showed that the Ar and the O_2 signal were ttenuated to the same degree hence it wa possibl to calcul t the tissue pO_2 because pAr in ti sue and air ar identical

Resting pO_2 was 57 8±4 6 and pCO_2 39 9±1 2Torr (±SE) At exhaustion during tati exer ise t 100% 50% and 25% MVC pO_2 wa 20±4 4 13±4 5 and 14±3 0 Torr whil pCO_2 wa 50±3 0 57±2 5 and 53± 1 7 Torr Recovery started ithi few sec after exercise for pO_2 whil pCO_2 continued to increase for another 50-60 sec up t 70-90 Torr Resting alue wer obtained after 10 min The resting oxygen consumption rate was alculated to 1 83±1 2 ml/kg/min (±SD) from the decrease in pO_2 during esting occlusion In thi situation pO_2 decreased to zero or lose to ero within 200 sec If then stati contraction was performed no further change wa seen in pO_2 whil pCO_2 increased t level ff t 80-100 Torr Thi increase in pCO_2 when no oxygen i consumed i an indication f pH change pH tending to f ll thus liber ting CO_2 which i buffered in the tissue

The ew technique here p sented i promising f the furthe study of metaboli event in the muscl It give liabl results ithin the range expected for pO_2 and pCO_2 but an also be used f the study of any the ga tension

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C 7

The effect of crown ethe on the ionic curr nts n the myelinated nerve memb ane

F ARHEM & H. KRISTBJARNARSON

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The properti of ionophor have mainly been studied on rtificial memb an (Haydon 1970) Very little is known of thei eff cts on natural excitable membrane Our aim with the pr ent study was to analys the effects on such natural membrane, namely the memb ane at the ode of Ranvier

W studied the effects on the ioni current in fibres from the clawed toad Xenopus laevis by potential lamp method (Dodg & F ankenhaeus 1958). A numbe of ionophors we tested but only the crown ethe dicyclohexyl 18-crown-6 was found to chang permeability paramete

The most striking effect (in the concentration range 1 100 µM) was an inactivation of the potassium current sociated with pos tive potential st p. The potas um current dec eased exponentially with time constant of about 10 ms concentration of 10 µM.

The effect di ppeared gradually aft epola risation to the holding potential. The time to reach 50 % recovery of polari ation t 70 mV was about 100 ms oncentration f 10 µM. The recovery time did not depend markedly on the holding potential

The ffect depended cl arly on xt rnal $[K^+]$ In t st solutions with high $[K^+]$ the inactivation wa pronounced than in olutions with low [K] The d crea of the outward pota sium current was proportionally equally larg the decreas of the inward pota sium cur ent oci ated with epolari ation fter positive potential tep. Thus ectification was observed.

Som of th se finding may be f levanc f the knowledg on the potas ium transport syst m in the rve membran It ften sumed that thi y tem onsists of long pore with g ting mechanism t the axoplasmi ond (Armstrong 1975) The main onclusion from the pr ent tudy i that our sults d not pport such impl mod l of the potas ium y tem.

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C 8

Inhibition of inspiration by low thr hold lung stretch eptor

A.T DiMARCO C on EULER J R. ROMANIUK & Y YAMAMOTO

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Vagally mediated excitatory effects on phren nerve activity elici ed by rapid inflations and d flations hav been epeatedly described. In addi tion to the facilitatory ffect of larg increa es in flow (1), it ha been ently ugge ted that volume dependent excitatory effects als exist (2, 3). Experiments we performed in 11 ana thetized cats (pentobarbitone PB) to stablish and furthe char ct rize the eff ct of varying volume feedback on inspiratory activity in the phrenic (P) and external intercostal (EI) nerve P and EI, tidal olume (V) flow ($\dot{V}$) tr cheal pressure (TP) and P_ACO_2 were simultaneously recorded. Int g ted P and EI wer empared with and without afferent f edback from lung movements in spontaneously b athing animal (tracheal occlusion) and with varying feedback in paralysed cat ventilated with servo- espi rator governed eithe by phr ni activity at different gains or by phreni trigg red ramp in flation of varying lopes and amplitud The computer averag of 5 test b ath wa compared to the averag of 5 preceding b aths

In lightly anaesthetized cats, aft varying delay from the onset of inspi tion, olume chang es elicited an excitatory effect on both P and EI. The chang in P EI from control was linea ly lated t change in TP and V but not to $\dot{V}$ This inspiratory facilitation ould be evoked by mall volum increments at unchanged value of $\dot{V}$ The ffect occurred ontinuously in every breath. Small additional dos of PB (4-6 mg/kg) abolish ed these excitatory effects without impairing the vagal inhibitory effects on inspiratory duration (T_I) nor the inspiration facilitating effect of pid inflations Although vagotomy liminated th volum facilitat ry effect excitatory ff cts could be produced in EI by lectrical vagal stimu lation at imilar thre hold trengths equi ed t produc ffects on T_I T produce excitatory ffects on P larger stimulus strengths w quired. P and EI usually ponded quantitatively diffe ently to the sam volume timuli in that any given volume chang produced larg lativ change in EI than n P Aft vagotomy EI wa often conside ably edu ed whe as P wa la tively les affected.

These ults sugges that volume elated in formation from low thr hold lung mechano p- tor can exert an excitatory effect on inspiratory motor neuron output. Howeve this facilitating ffect probably doe not involve the R_β neurons in its entral pathway doe the inhibitory flex.

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C 9

Afferent A and C fibre interaction in the baroreflex

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Input from art rial baroreceptors are transmitted to the brain via myelinated (A) and non myelinated (C) fibres The A and C fibres differ in receptor thresholds and discharge frequencies but reflex responses to activity in the fibres are roughly the ame: bradycardia and a fall in arterial pressure The aim of the present study was to examine wheth r afferent signals in A and C fibres show interaction in their reflex effects or whether th y are subject to simple addition

The rabbits were anaesthetized with chloralose and ureth ne With other baro receptor nerves intact the central end of the cut left aortic nerve was stimulated with two sets of electrode one of excit ation of A fibres (pulses of 60 100 Hz 0 02 ms and 6 V) and one for activation of C (and A) fibres (2 4 Hz 1 ms 6 V) The pul e rates in C timulations were too low to voke eflex effect via A fibr Several timulation serie consisting of sepa rate A and C fibre timul tions and a com bined stimulation wer run in each animal Pulse frequencies were var ed from one series to another in order to obtain a range of low to medium reflex esponses

The hypotensive response to combined stimulations of A and C fibres (A C) was found to exceed the sum of responses to separate A and C stimulations in 21 of 22 series For whole-nerve activity in the left renal nerve a greater than additive effect was observed in 16 of 24 series (P 0 01) while in six of the series the responses were equal Median values of the ratio AC/(A+C) were 1 28 and 1 11 respectively Similarly C stimulations which in comparison to A stimulations affected sympa thotic activity to the kidney relatively more than blood pressure in 13 of 16 series (P = 0 01) produced a greater sympa thotic inhibition when added to a back ground of A fibre activity than when alone Changes in heart rate were minimal

Others have found a positive interaction between reflex responses to stimulation of all fibres in ipsilateral aortic and carotid sinus nerve (Kendrick Matson & Lalley 1979) The pres nt results show that a syn rgi tic interaction may exist also between reflex responses to activity in dif ferent fibre populations within one baroreceptor nerve

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C 10

INFANT LOCOMOTION
an inherited motor prog am for locomotion?

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Throughout the animal kingdom it has been shown that locomotion is generated by central motor programs genetically encoded for each species (eg Wilson 1961 Grillner & Zangger 1979) An hierarchical organization has been revealed i mammals as a significant part of the central networks is located in the spinal cord (Brown 1911 Grillner & Zangger 1979) and as the spinal networks together with reflex interaction can produce a locomotor pattern that even n many details resemb les that of intact animals (Forssberg et al 1980)

There is today no possibility in a direct way to explore how the neural control of locomotion is organized in humans but the aim of the present investigation is to study the development of human locomotion to see if it is in accordance with similar central and hierarchical organization The human starts to walk without external support about one year after birth but already at birth locomotor-like activity can be induced if the infant is held erect with the feet on a flat surface (Andre-Thomas & Autgarden 1966) This inhe ted locomotion has been analyzed in detail in 4 infants by recordings of movement muscular activity and force reactions The locomotion is irregular and compared to adult locomotion there are significant differences such as a pronounced hip flexion a variable digiti or plantigrade foot contact a premature activation of the calf muscles (before foot contact) and a high degree of cocontraction between antagonistic muscles When the children learn to control their locomotion (ie walk independently) the pattern succesively develops to be more like the adult locomotion but still can the variations from the infant locomotion be seen (see also Statham & Murray 1971)

Of special interest is that certain of the differences are similar to those seen when the adult bipdeal gait is compared to the quadrupedal one in which only the toes contact the ground (digitigrade). It is therefore suggested that humans are born with an innate network for locomotion that is programmed fo a quadrupedal gait The ontogenetic development should then reflect the phylogenetic evolution to a bipedal gait due to modifications of the central motor prog am

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Fractional and morphological evidence that small resistance vessel in human omental have littl adrenergic innerv tion

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The quantity of adrenergi innervation of arteries varies between different regional circuits as well as between consecutive vascula segments (F lkow & Neil 1971) We have investigated the amount f adrenergic innervation of small human arteries (internal diameter i d ≈ 200 µm) from the omentum as it has been shown earlier (Stjarne & Brundi 1976) that larger arteries i this circuit (internal diameter ≈ 1 1 5mm) have adrenergic innervation

Segments of 19 human omental arteries were mounted on a myograph which permits precis measurements of wall tension whil the internal circumference can be controlled (Mulvany & Halpern 1976) After mounting the media thickness was measured by light microscopy (9 60±0 50 µm) The wall tension response to K-depolarisation Δ T_K was measured (2 02±0 15 mN/mm) and the magnitude f this response was not affected by phentolamine (1 µM) Cumulative noradrenaline (NA)-dose-response curves showed NA-ED of 0 415 µM (NA-pD =6 38±0 30)and a maximum response t 4 µM NA of 1 86±0 15 mN/mm This maximum respons corresponds to an active media stress of 198±20 mN/mm² which is an indicator of the viab lity of the preparation The NA-response was totally nhibited by phentolamine Add tion of 3 µM cocaine (nhibit ng the amine pump i any adrenergic nerve-terminal) had no sign ficant influence on the maximum respons nor did it produce any significant shift of NA ED (ED_5 ratio=1 16 0.31) Simila experiments using two large omental arteries (i.d 518 µm and 752 µm) showed a greater wall tension response ($ΔT_K$=4 95 mN/mm and 10 44 mN/mm) and a shift of the NA-ED to smaller values with cocaine (ED -ratio 2 20 and 1 77) On completion of the mechanical experiments some of the vessels were prepared for fluorescence microscopy This showed that the "200-µm vessels had very little or no innervation whereas the two "large vessels were richly innerv ted As further control mesenteric arteries (i d 150-200 µm) from Wistar-Kyoto rats were invest gated These vessels showed very rich innervation with fluorescenc icroscopy as well as a cocaine-induced left shift of the NA ED (ED -rati ≈ 3) and considerable reduction (about 50%) of the K-response with phentolamine

The lack of a left shift f NA-ED with cocaine indicates a very low amine pump activity i the vessel wall The lack of ffect of phentolamine on the K response indicates very littl or no neural release of NA during K-depolarisation Thes findings together with the scanty fluorescence of the "200 µm omental vessels give functional as well as morphological basis to conclude that these vessels have very littl adrenergic innerv t on It i therefore unl k ly that these ves l play a ignificant role i the sympathetic control of blood pressure

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C 12

β-adrenergic d l tor teraction with the const rictor response re tance vessel f sk and skeletal muscl during hemorrhage

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Much effort ha been devoted to the problem of regulation dur ng hemorrhage of peripheral vascular resistanc hemodynamically important vascular area such a sk and skeletal muscl The interest however ha lmost exclus vely been focused on the socenstrictor mechanisms brought into action i response to bleeding l ttl attention being paid to the po s bility that anta goni ti dil tor i teract on with the vasoconstri tor influence may be of gn ficance i determining the net res stance effect The present tudy describes quite pronounced β-adrenergi d lator influence i k and sk letal muscl during reflex sympatho-adrenal act tion evoked by graded hemorrhage The dil tor effect wa revealed i terms f much greater increase of vascular re istanc i the scul bed with blocked β-adrenoceptors (propranolol) than the vascular bed i which the adrenoceptors were left intact I both ti sues the β-adrenergi dilator influence was graded i rel tion to the level of hypotension and t severe bleeding (hemorrhagi hypotension f 50 mm Hg) vascul resistanc was usually bout twic a l rge fter than before β-blockade In some preparations the β adrenerg c effect wa fact much more pronounced muscl s well s i k so that blood flow lmost eased after adm t a tion of propranol l

The β-adrenerg d lator mechan sm may erve to improve nutritional blood flow by counteracting the α-adrenerg constrictor response Further t may contribute to determine the di tribution f cardiac output dur ng reflex sympatho-adrenal activ t on

C 13

HYDRAULIC CONDUCTIVITY OF THE PERITUBULAR CAPILLARY MEMBRANE IN THE RAT KIDNEY

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The hydraulic conductivity of the pe itubul capill ry membrane was det rmined from 1 The si gl nephron fluid bsorption and 2 The net dri ing f rce i e f om the hydr taric and colloid smo-ti pre sure in enal int rstitium and pe itubu-lar apillary blood as investig ted with micro-punctur t chnique and comp t r-b sed mod l

During control antidiur tic conditions the net driving f rc was timated t 15 4 mm Hg and the hyd li onductivity t 1 04 nl/(mi mm Hg) pe 100 g at During 4 % and 10 % saline xpan sion the net drivi g f decr ased t 12 5 mm Hg and 6 4 mm Hg e pectively whereas the onduc ti ity increased t 1 83 and 3 14 nl/(mi mm Hg) pe 100 g rat The reduction i net dri ing f rc was thus at le t partly compensated by an in-reased hyd aulic conducti ity In the glomeruli the net driving f ce f r filt tion incr ased from 14 2 mm Hg during contr l conditions t 21 2 mm Hg and 25 3 mm Hg during 4 % and 10 % aline expansion wheres the correspondi g hyd li onducti ity decreased from 1 13 1/(min mm Hg) per 100 g t t 1 03 and 0 80 nl/(min mm Hg) per 100 g t during the two expansions

Thes hange in the hydrauli conducti iti will cause ret rded sor tion f the xc s fluid during line xpansion but then lso prolonga-tion of the time f the filt ri g proc s t

ope te This is in favour f r the renal elimina tion f toxic substances and metabolic end-products. From the mass t an port of albumi and inulin the effe tive pore radius was cal lated to range between 50–70 Å Using the Poiseuilles equation the perme bility term A /d i the total pore are over the pore length could be evaluated The e-sults show that the tot l po are i very large at about 1 % f the total apillary area During 4 % and 10 % aline xpansion the tot l pore ar a will increas to 1 8 % and 3 2 % for the two ex-pansion re pe ti ly It is supposed that the in-creased total pore a a duri g expansion is caused by the str tchi g f the vessel wall which in turn i due t an i ease i the t ansmur l pressure diff renc from 8 6 mm Hg during control conditions t 12 8 mm Hg during 10 % saline xpansion

C 14

Cer b al sodium/angi ten in inte a tion tudi d by RIA d te mi tion of u in y a ginine v pres in

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Angiote in II (A II) infus d int th th d o l ta al cer b al t icl of g at has be n found to inter ct with ce brospin l fl id (CSF) N in ll iting thir t rel s of a tid reti h rmon (asopr in) n tri i and l ation of th arterial blood pr r (cf And on 1977). A garde th antidi reti and dip og ni ff t th int ra tion ha als be d mon t ted with a bth hold (p r in ff ti) amounts of A II (Lek ll & Rundg 1977) However s f th e id co f r a m tu l potentiation betw l ted CSF Na nd A II has m ined ind t.

To obtain a direct quantitati me re of th c reb l Na/A II inte ction inf ion (0 02 ml/ min) f a) 0 25 M N Cl (N 10) b) A II (0 3 g/kg min) in isotoni NaCl (N 10) and) A II (dos as b) in 0 25 M N Cl (N 9) were made int th l toral ce ebral ntricl of two hydr ted g t During th pre inf sion cont ol period a ginin a opr in (AVP) was ot detectabl in th in with the m thod mpl yed.

The m an (±SEM) amounts f AVP re ove d by radioimmuno say in the urine in re pon t th v rious inf ion were l f ion a) 2.8 ± 0 7 ng b) 3.6 ± 1 3 ng and) 13 3 ± 2 2 g Th th AVP r pon e to th simultaneou l f ion f hyp to NaCl and A II wa pp oxim tely two tim a l rg s the sum of the re ponse to the pa at inf ion of hypertonic NaCl and A II gg ting a m tu l Na A II pot ntiation re g d tim lation f th ne ohypophys al p al el a e l f ion) inv l bly licited thi t in th prehydr t d animals wheres th inf i f m rely A II of 0 25 M N Cl did not ha any d pe og i ff t.

The ults p ovide f th upport f r the idea (cf And on 1978) th t le at d CSF N con ce t tion and angiote l II ll it AVP rel a nd thi at by a ting pon th am kind of juxta cereb ove t icul r re pto s

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Developmental patterns f PGE_2 and 5-HT metabolism in isolated perfused f tal and neonatal rabbi lungs

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Pulmonary circulation i known to metabolise selectively vasoactive hormones (Bakhl & Vane 1974) Very li tle is known about the development of these processes in f tal and neonatal pulmonary circulation. The aim of the present study was to investigate the development of PGE_2 and 5-HT inactivation in fetal and neonatal rabbi lungs

Lung perfusions were made at 28th 30th and 31rd days of fetal lif and on the firs second and seventh day after birth. Fetal rabbits were obtained by caesarean section during spinal anaesthe ia and the offsprings were allowed to breathe spontaneously In preparing lung perfusions pentobarbital anaesthesia was used After thoracotomy the ani mals were heparinized i ductus arteriosus was ligated and plastic cannula was inserted into the pulmonary artery through the righ ventricle The pulmonary vascular bed was washed with 1 ml of saline, and the isolated lung block was connected to perfusion apparatus Perfusions were made with Krebs bicarbonate (pH 7.4 37°C) containing 1 g/l glucose at flow of 8 ml/min and perfusion pressure was maintained within physiological limits Measurement of injected PGE_2 (200-300 ng) was made by bioassay with hamster stomach strip and f infused C^{14} 5-HT (0 4 µM) by column chromatography as described ea lier (Bakhl Hartiala T ivonen & Uotila 1979)

The survival f PGE_2 presented decrease from 11 % at 28th day f gestation 4 3 % birth and this level remained constant during the first pos natal week The metabolism f 5-HT on the other hand remained rel tively tabl up to firs postnatal day when 43 % f infused serotonin was metabolized whereas on the 7th day af er birth i was decreased to about 30 %

The results demonstrate the prostaglandin and amine inactiva ion develops differen ially during f tal and neonatal period in rabbit lung Compared to our earlie resul on the development f lung lipoprotein lipase i perfused rabbi lungs (Ni tanen Simberg & Hartiala 1979) it i inter es ing to note tha PGE_2 metabolism and lipolysis were increased wi h age However lipolysi expressed peak immediat ly preceeding birth not seen wi h PGE_2 Consequen ly it is obvious that pulmonary metabolism i regulated by vari ty of factors i fetal and neonatal periods

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C 16

Effect of cholera toxi on serotonin content of enterochromaffin cell i the cat small intestine

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Cholera toxin (CT) instilled the small intestine causes massive secretion In accordance with the hypothesi that seroton (5-HT) may be the diarr hoeogeni messenger it ha been proposed that 5-HT may be involved the secretory response f the intestine to CT (Fujita et al 1974) Since most of the intestinal 5-HT is local zed to the enterochromaffin cell (EC) the effect of CT on the 5-HT content of EC in the cat small intestine was studied

The experiments were performed on adult chloralose-anaesthetized cats with bilaterally denervated adrenals Following preganglioni splanchni denervation segments of the jejunum were isol ted. Crude extract of CT or heat inactivated CT was instilled in the segments for 1/2 h and then removed Intestinal fluid transport was continuously recorded. After 1 to 5 h,specimens of the segments were removed frozen in liquid propane freeze-dried and treated according to the Hill rp-F lck procedure Sections of the specimens were studied by fluorescence microscopy and the fluorescence intensities of individual EC in coded sections were measured i a Leitz MPV II cytofluorimeter From each specimen the fluorescence intensity of 20 randomly s lected EC were recorded (cf Ahlman 1976; Pettersson 1979)

In cats where a typical secretory response to CT was observed the cytofluorimetric measurements revealed a ignif cant (p <0 005) decrease by about 70% in the fluorescence intens ties of EC CT-exposed segments as compared to 0 h control segments In 5 h control segments the decrease was about 25-30% The CT induced decrease 5-HT fluorescence was observed i EC located both i the crypts and on the intestinal villi The magnitude of the decrease was greater than that observed (about 30%) fter vagal o plenchnic nerve stimul tion for 10-60 min (Ahlman 1976; Larsson et al 1979) In cats where no typical secretory response but rather absorbtion was observed despite the CT installation the 5-HT fluorescence of the EC did not d ffer significantly from 5 h control segments those observed i cats exposed to heat inactivated CT

These res lts demonstrate that the physi logical response to CT is well correlated to a marked decrease f 5-HT content i intestinal EC, and support the hypothesi that 5-HT may be involved i the secretory response to CT

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C 17

Ca^{2+}-sequestration by microvesicles i ola ted from bovine neurohypophy s

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Nerve endings from the neurohypophysis contain microvesicles but their function has not been established In the present study purified microvesicles with and without coats were isolated from bovine neurohypophyses using the proc dure of Blitz et al 1977 The purified microvesicle were found to contain Ca^{2+} dependent ATPase activity and to be able to accumulate Ca^{2+} in the presence of ATP and Mg^{2+} The purified microvesicles were imilar to brain microvesicles a judged by electron microscopy and SDS-electrophoresis in polyacrylamide g ls As judged by SDS polyacrylamide gel electrophoresis their protein composition was very different from neurosecretory granul In terspersed betwe n the microv sicles was a b ckground of irregul r patches and lattice of hexagons and pentagons compatible with the structure of isolat d coats

The microve icles contained Ca^{2+}-dependent ATPase activity The activation by Ca^{2+} of the enzyme could be seen at Ca^{2+} concentrations down to 10^{-7} M fr e Ca^{2+} (i the pre sence of 2 mM Mg^{2+} and 1 mM ATP) The spe cific ATPase activity w s 20 µmol/mg protein per h Pos ible ATP hyd olysis by contami nating Na^{+} K^{+} ATPase activity i not likely since ouabain was added to the medium

In the presence of ATP and Mg^{2} the mi crovesicles accumulated Ca^{2} Addition of the ATP analogue AMP P-C P instead of ATP caused no Ca accumulation Oxalate (10 mM) stimulated Ca^{2+} accumulation and the Ca^{2+} ionophore A23187 inhibited the accumulation Under optimal conditions Ca^{2+} uptake wa 340 nmol/mg of protein (steady state) in the presence of oxalate The present data show that microvesicles imilar to those prepared from brain can be isolated from the neurohypophysis The microvesicles may have an important function in calcium sequ tration The data from SDS polyacryl amide gel electrophoresis support the hypothesis that these microvesicles are not etrieved membrane from neurosecretory granules that have emptied their contents

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C 18

Calmodulin a d ATP dependent alc um t an port n subcell l fra t on f om the ox-neurohypophy i

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In the neurohypophy is calcium ions (Ca^{2+}) mediate ecretion of v sopres i nd oxytocin (review see Thorn et al 1978) To maintain sensitivity cytosolic free Ca^{2} is held low at re t (pCa 6 7) by protein buffering and ATP-d pend nt seque tratio into intracellular organelles as well as expulsion across the pl ma membran W have studied the effect of the c lcium dependent modulator protei c lmod li (reviewed recently by Ch ung 1980) on ATP-depe dent $^{45}Ca^{2+}$ uptake into subcellul r fractions from the ox-n urohypophy i In microsomal fraction (Rus ell & Thorn 1975) pretreated with EGTA (10 3 mol/l) to emove endogenous modulator c lmodulin max mally elevated $^{45}Ca^{2}$ accumulation fou five fold at an incubation pCa of 7 0 in a medium (pH 7 0) containing (in mmol/l) KCl 130 TES 20 $MgCl_2$ 2 Na_2ATP 2 $^{45}Ca^{2}$/$CaCl_2$ [0 05 Ci/mmol] 0 2 EGTA 0 5 30°C Th amount of calmodulin half-ma imal fo the effect was about 10 µg/500 µg membr e protein In this membrane fraction ATP-depe dent Ca^{2+} uptake and passive efflux re i sensitive to cAMP We are extending thi study to include more cle rly d fined membrane fractions

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C 19

Synapti activity in lumbar spinal motoneurones f embryonic chickens

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Fig 1 Intracellular record from GL motoneurone to graded timul tion f the GL nerve Superimposed sweeps Chick 21 days

The chick has been used extensively in anatomical studies of neural development We have now begun explor the development f synapti activity in lumbar spinal motoneurones during the last days before hatching using intracell lar recording
Chicks aged 19-2 days were decapitated and immobilized with d-tubocurarine The lumbar region f the spinal cord (segments 25 30) was exposed through window in the shell Various branche f the sciatic nerve were cut peripherally and isolated timul tion Intracellular ecordings f synapti activity was made using standard echniques Microelectrodes had resistances f 30-80 MΩ. ecordings could be made in which the resting membrane potential (60-70 mV) and action potential (ca 100 V) remained stable for upto 2 5 h or more Motoneurones innervating the lateral gastrocnemius (GL) muscl were identified by activating the cell body antidromically on stimulation f he muscl nerve In addition to the antidromi action potential GL motoneurones responded to graded stimulation f the GL nerve with graded early (6-8 msec) epsp The fibular ibial and sural nerves gave only later usually more variabl and predominantly inhibitory synapti effects (Fig 1) The homonymous early epsp was ften li ited by stimulus intensities subthreshold for antidromi activation of the motoneurone and was usually maximal t three time the intensity required to activate the motor axons The epsp was educed in amplitude by depolari ing curren i jected into the cell but was unaffected by hyperpolarizing current F ired or repetitive (50 Hz) stimulation caused depr ssion f the amplitude f the epsp
We suggest tha the early epsp f GL motoneurones in the chick ediated by chemical synapses and i analogous to the homonymous monosynapti epsp obtained in mammalian motoneurones i esponse to timulation of low-threshold muscl afferents

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C 20

SUMMATION OF DENDRITIC SYNAPTIC POTENTIALS IN HIPPOCAMPAL PYRAMIDAL CELLS

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A conductance-opera ing ynapse may act as shunt f neighbouring synapses (Burke 1967) We wanted t see wheth such synaptic inte ference exi ts f cortical pyramidal cell

The transverse hippocampal lice prepara tion llows selective ctivation f fferent input fibres The pyramidal cell lie with thei apical and basal dendriti tree pa ll l to the surf ce f the lice Afferent fibres lso run parallel to the lice surface but perpendicula ly o the dendri l t es Cuts long th dend i tic axis may be used to restrict the fferent nput to small and identifiabl part f the dend iti tree I the pres t tudy lesions re t icted the ff ent input to two b idge f fibre lying t diff ren distances from the cell body

The pyramidal cell we acti ted orthodromically with subthreshold current so that pure EPSP as evoked A timulation sequence con i ted f three diff ent timuli Fi t timul tion of the afferents running through the proximal site second the distal g t and third both input togethe The EPSPs from numbe f such timul ti sequence wer veraged f ach ngl and for the double input The esult from th imul taneous timul tion was then compared to the algebrai um of the EPSPs from the activation through each gat alone

When the cell was k pt t its resting potential the lgebrai um was more prolonged in 85% f the cell than the EPSP due to the double input When the cell was hyperpola ized however the EPSP resulting from the two types of ummation were identical suggesting linear ummation f the EPSP due to the proximal and di tal inputs The non-linea umma tion t the depolari ed memb an potential was probably due to an interf rence by the IPSP whos effect was emoved by hyperpo l ization The linear ummation t the hyperpol ri ed membrane potenti l wa see in 15 of 16 cell

I conclusion linear ummation f EPSPs f om neighbouring excitatory synapses occur at a hyperpolari ed memb ane potential Thi might indicate th t the syn pse do not i terfere with ach the Howeve definite conclusions must await experiments whe the IPSP i pharmacologi lly blocked

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C 21

Pharmacological effects on sleep in the immature animal

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As studies on adult cats now indicate that divergence in either direction from an optimal level of noradrenergic (NA) activation inhibits paradoxical sleep (PS) (Putkonen 1979), and as the balance between NA and 5-HT brain stem systems seems to be crucial in sleep regulation we have studied the effect of pharmacological manipulation of the NA 5-HT and acetylcholine systems on sleep in kittens In these, quiet sleep (QS) is virtually absent at birth, and monoaminergic regulation is claimed to have very little role during the first postnatal weeks (Adrien 1978)

We have made day-time polygraphic recordings from kittens between 6 days and 2 months postnatal age during 8 hrs after i p drug injection with the kitten separated from its mother

Most effects were as in adult cats already from 1 week of age While clonidine (CLO) 10 µg/kg inhibited PS phentolamine (PHE) from 5 mg/kg increased PS by increasing episode duration and the probability of PS directly after waking PHE 20 mg/kg decreased QS At 1 month the larger dose was less effective although it still antagonized CLO

Yohimbine (YOH) increased arousal and state transitions and 2 mg/kg antagonized CLO The postsynaptic blocker prazosine (PRZ) 1 mg/kg increased PS episode durations but lowered the PS incidence At 2 weeks maximal PRZ effect was obtained with 0 5 mg/kg, but from 1 month with 2 mg/kg PRZ always potentiated the effect of CLO

Citalopram (CIT) 5 mg/kg, which inhibits 5-HT uptake inhibited PS already at 1 week as in adults In PCPA treated animals CIT tended to normalize sleep

Atropine (ATR) 2 mg/kg inhibited PS and increased QS from 1 week of age ATR 0 075 mg/kg slightly shortened PS episodes but in combination with PRZ (20 mg/kg) inhibited PS radically

The results show that sleep regulation in kittens depends on similar systems as in adults although the characteristics of QS are not fully developed The differences are mainly quantitative and the sensitivity to the studied agents changes during development

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C 22

Metabolic consequences of membrane depolarisation

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In excitable tissues membrane depolarisation is mainly an electrical event and the depolarisation in itself initiates the next depolarisation and by this the propagation of the impulse.

However a series of non-excitable tissues also depolarise when they are stimulated These tissues include brown adipose tissue and it has been found that the resting membrane potential of 50 mV is diminished to 25 mV when the tissue is stimulated by norepinephrine and thermogenesis is activated The consequences and function of this depolarisation have however remained unknown

We have recently demonstrated (Al Shaikhaly Nedergaard & Cannon 1979) that brown fat mitochondria possess a mechanism for Na stimulated Ca^{++} release

We have now found that this mechanism allows the mitochondria to regulate the extramitochondrial (in the cell i e the cytosolic) steady state Ca^{++}-concentration accurately in the range 0 3 1 µM dependent on the cytosolic Na-concentration This steady state Ca^{++} level is most sensitive to changes in Na -concentrations in the range 5 15 mM

We have also found that it is possible by this mechanism to regulate the activity of the mitochondrial glycerol 3-phosphate dehydrogenase (which is Ca^{++}-dependent) This enzyme plays a key role in regulating cytosolic concentrationsof glycerol 3 phosphate and an increased activity of the enzyme will help channel activated fatty acids towards combustion instead of towards (re-)esterification

Based on these experiments and on the sensitivity of norepinephrine stimulated respiration (but not lipolysis) to extracellular Na^{+} we suggest that the metabolic significance of membrane depolarisation in non-excitable tissues may be related to a Na^{+}-dependent increase in cytosolic Ca^{++} with regulatory functions as a result; in brown fat these may involve the control of the reesterification process

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C 23

Influence of LH and FSH on the adenylat cycl activity in granulosa cell isolat d from preovulatory rat follicles

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Preovulatory granulosa cells isolated from the ovaries of HCG reated immature rat responded t FSH as well as LH in vitro with increased cAMP f r- ation, although FSH wa more effective (Hamberger al, 1978) The aim of he present study was to explore whether this stimulation of cAMP f rmation was correlated to an enhanced adenylat cyclase activity in these ll

Preovulatory granulosa cell were isolated as described by Nordenström et l 1980 Preparation of membranes and he assay for adenylat cyclase activity was performed with mino modifications described by Khan and Rosberg (1979) Each sample contained membranes f om granulosa cells from 8-10 follicles

The adenylate cyclase acti ity was significantly stimulated by low concentration (0 01 g/ml) f either FSH or LH. A concent ation of 1 μg/ml f FSH and 50 μg/ml f LH was needed to elicit maximal response When GTP analogue (Guanylyl-imidodi phosphate) was added to the adenylat cyclase assay there was 10-fold increase in he enzyme activity although the relative imulation of FSH and LH was not ltered.

These result are in good accordance with the findings that both LH and FSH in vi stimulat cAMP formation in solated preovulat ry granulosa cell This lso indicates hat the increased cAMP formation depend on an effect f the gonadotropin on he receptor-adenylate-cyclase level in the granulosa cell membrane Furthermore it sugges higher sensitivity f these cell to FSH as compared t LH

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C 24

Decreased receptor binding affinity of mono-^{125}I-gluc gon whe Met^{27} i oxidi ed during the iodination

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Iodoglucagon i widely used in radioimmunoassay and bi logical work as tracer for the 29 amino acid polypeptide hormone glucagon Until ecently we iodinated glucagon using the chlo amine-T method (CHL-T) However HPLC chromatography revealed that the sulphur of Met^{27} was oxidi ed This oxidation did not occur when glucagon was iodinated ing the lactoperoxidase method (LPO) f oxidation (Markussen & Larsen 1980)

Glucagon was iodinated by CHL-T (Jørgensen Larsen 1972) (specifi acti ity 319 Ci/g) by LPO (Thorell Johansson 1971) (specifi acti ity 322 Ci/g) The specifi binding (defined total binding (tracer only) minus unspecific binding (trace plus urplus f unlabelled glucagon)) f thes two trace p epa tions to isolated t dipocytes nd hepatocytes measured as described (Gliemann Sonne 1978 Sonne et al 1978) When the concentr tion f trace 1 K_d for the binding f glucagon the ati f bound hormone/f ee hormone (B/F) become ident cal with the ti concen tr tion f eceptors (R_o)/di soci tion con ta (K_d) f the labelled ligand Therefore when carried out the same cell pool (i same R_o) variations in B/F reflect vari tions in K_d

Only half as much ^{125}I-glucagon labelled by CHL-T was bound compared to the trace labelled by the LPO method (Tabl I) indicating that the C terminal part f the gl cagon molecul is f importance f the interaction with its ecepto Similar results were obtained on two the batche f tra cers

Tabl I Binding ffinity f ^{125}I-glucagon labelled by the CHL-T the LPO method
^{125}I-glucagon (10 pM) was incubated 37°C for 30 min with 1 % (/v) adipocytes or for 60 min with hepatocytes (5x10^4 cells/ml) B/F indicates the value for the specific binding Mean of 5 replicates

^{125}I-glucagon	Adipocytes B/F 10^3	%	Hepatocyt B/Fx10^3	%
LPO	4 4	100	2 2	100
CHL-T	2 5	56	1 1	50

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C 25

Ipsi and contralateral cold sensitivity after unilateral lesions of the lateral spinal funiculus of the cat

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Behavioural thermosensitivity of the paws was investigated before and after unilateral surgical lesions of the lateral spinal funiculus above the 5 th cervical segment The sensitivity for skin temperature decreases was investigated independently for either body half on alternate days in an automatic T-maze The cats performance of the discriminatory task with either body half could be sustained whenever necessary by an acoustic cue which was delivered optionally in unison with the thermal cue

One cat received two subsequent lesions of the same lateral funiculus (1 o partial transection involving ventral half 2 o complete transection) In two more cats the whole of one lateral funiculus was transected immediately No lesion caused any change of the ipsilateral sensitivity The ipsilateral paws were used to discriminate equally small temperature decreases pre- and postoperatively i e 4° C or less Neither was any contralateral change found after the first partial lateral funiculus lesion of the cat who was operated two times

The complete lateral funiculus transections caused profound contralateral thermosensory disturbances in all instances There was a residual sensitivity however which in the best case consisted of an eventual ability to discriminate a temperature difference of 20° continuously above criterion level (22/32 correct responses/session) for a long period This lowered thermosensory capability perhaps was due to something else than a simple sensory attenuation because there was a lack of interaction between the body halves The above cat who was performing a coarse discrimination with the defective side was performing a much more difficult task with the other side on alternate days The performance on the defective side failed to improve for its simple task however when the task on the other side was made even more difficult and demanded an increasingly larger behavioural effort

It was found previously (Norrsell 1979) that unilateral lesions of the lateral funiculus caused contralateral thermosensory defects in the cat but the technique did not permit quantitative estimates Now it may be concluded that unilateral interruptions of the lateral funiculus cause drastic and purely contralateral defects of the cold sensitivity The defective side retains a residual thermosensitivity by having the means of being useful for discrimnation of large temperature differences but may lack normal thermal sensations The findings are most easily explained by supposing that the lesions interrupted a crossed ascending thermosensory pathway which is necessary for normal behavioural thermosensitivity

Reference

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C 26

Binocular activation of perigeniculate neurones in squint operated cats

G AHLSEN S. LINDSTRÖM & F S LO

Dept of Physiology University of Göteborg Swede

The perigeniculate cells serve as interneurones i recurrent inhibitory pathway to principal cells in the lateral geniculate nucleus Most perigeniculate neurones receive excitation from both eyes through collateral connexions from monocula ly activated p incipal cells W have now studied the binocular input to perigeniculate neurones in squint operated cats It is well known that such an operation at an early age leads to a disruption of normal binocula connexions from princi pal cells to neurones in the primary isual co tex

Three kittens were made squinting by sectioning of the left lateral (two kittens) or medial (one kitten) rectus muscle 21 days after birth The animal were studied about two years later in acute experiments We first recorded from 30-40 neurones in the primary visual cortex of each animal As expected for squint operated cats most cortical neurones were activated from eithe the left o the right ey nly 14% of th cells were found to have a binocular input

The perigeniculate neurones in the same animals seem to recei e completely normal binocular input. Of 60 identified cells 78% could be activated via both eyes as compared to 73% in normal control animals The distribution of eye preference was also the same in the squint operated cats as in the normal

The visual cortex (areas 17 and 18) was removed during the experiment i one squint operated cat. Binocularly activated pe igeniculate cells were found also after this lesion, indicating that the excitation was mediated in subcortical connexions as in normal animals Thus perigeniculate neurones seem to be able to maintai bi nocular connexions from principal cells inspite of a noncongruent isual input, while cortical neurones lose bi nocula connexions in this situation. This difference in plasticity at the two termination sites for the principal cells uggests that postnatal reorganization of synaptic connexions may be functionally specific and controlled by the postsynaptic neurone

DEMONSTRATIONS

D 1

Demonstration of a technique for evaluating recurrent inhibition in man with on-line computer analysis

H HULTBORN R MACKEL & H WIGSTRÖM

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A method that is based on paired Hoffman reflexes has recently been developed to estimate recurrent inhibition in man (Bussel & Pierrot Deseilligny 1977) This technique has later been used to deduce the supraspinal control of Renshaw cells during various ankle movements (Hultborn & Pierrot-Deseilligny 1979) The data obtained are subjected to on-line analysis by a digital computer (Nord-10/S) to provide the necessary guidance for the following steps in the experiment Peak to-peak amplitudes of the evoked EMG signals are measured within several adjustable time windows and the results are displayed in diagrams on a graphic screen Triggering of the stimulating sequence is controlled by the computer which is useful for instance in the analysis of voluntary tracking movements

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D 2

Evidence that the non-reciprocal inhibition of spinal motoneurones by Ia muscle spindle aff rents and by Ib tendon organ afferents is mediated by the same interneurones

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Recent observations ha e shown that impulses in Ia muscle spindle afferents may cause inhibition of motoneurones of synergist as well as of antagonist muscles (Fetz et al 1979 Jankowska et al 1980) They showed also that the pattern of distribution of the Ia non-reciprocal inhibition closely resembles the pattern of Ib inhibition (Jankowska et al 1980) The possibility has now been considered that the same interneurones mediate the Ia non-reciprocal and Ib inhibitory actions and in order to test this possibility the following experiments were performed Di and trisynaptic IPSPs evoked by Ia and Ib afferents of triceps surae were recorded in a number of motoneurones of plantaris flexor digitorum longus and quadriceps muscles Ia afferents were activated by adequate stimuli (brief triangular stretches of 10-20 µm which were below threshold for Ib afferents cf Fetz et al 1979) Ib afferents were excited by electrical stimulation of the nerves to the same muscles using technique of Coppin et al (1970) The technique consists in raising the threshold to electrical stimuli for Ia afferents above that for Ib afferents by a prolonged vibration of the muscles and allows selective activation of a certain proportion of Ib afferents A comparison was then made of IPSPs evoked by Ia and Ib afferents stimulated separately and by properly timed stimulation of both of them Spatial facilitation of effects of Ia and Ib afferents has been disclosed in a great proportion of the analysed motoneurones indicating their mediation by common interneurones (cf Lundberg 1979) Our results show thus the existence of a shared pathway of Ia non-reciprocal and Ib inhibition although they do not preclude the possibility that inhibitory actions of muscle spindles and tendon organs are evoked via both common and separate pathways

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Studies on the non-adrenergic non-cholinergic innervation of the stomach of the rainbow trout (Salmo gairdneri) using "Lomni -preparation"

S. HOLMGREN & S. NILSSON

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The vagal parasympathetic innervation of the stomach of the rainbow trout is inhibitory causing relaxation of the stomach followed by "rebound" contraction shortly after the end of stimulation (Campbell 1975)

The present experiments are performed on saline perfused stomach measuring the intragastric pressure as well as the perfusion flow. The outflowing perfusion fluid is directly used to superfuse an isolated strip of gastric smooth muscle, mounted in paraffin oil (see figure).

Stimulation of the vagus nerve to the whole perfused stomach produces small inhibition of the intragastric pressure during the stimulation followed by large "rebound" contraction of the stomach and simultaneous reduction in flow rate ... after cessation of the stimulation.

The unidentified substance (transmitter) released in the perfused stomach by nerve stimulation superfuses the isolated strip producing the same response of the strip: an initial small relaxation (or inhibition of spontaneous activity) followed by pronounced contraction. The effect can be mimicked by direct transmural stimulation of the isolated strip. The responses to transmural stimulation can be blocked by tetrodotoxin indicating an effect i.e. nerve mediated release of transmitter.

The responses of both whole stomach and strip are not blocked by atropine, hyoscine, phentolamine or propranolol, indicating the presence of non-adrenergic non-cholinergic type of innervation of the stomach of the rainbow trout (see also Grove & Campbell 1979)

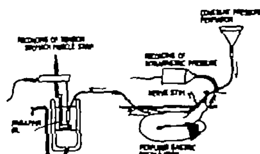


Figure. The "Lomni -preparation"; perfused whole stomach and an isolated stomach muscle strip superfused by the outflow from the whole stomach

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EFFECTS OF HYPOXIA AND HYPERCAPNIA ON CENTRAL NOREPINEPHRINE (NE) NEURONS AND ON PERIPHERAL SYMPATHETIC ACTIVITY

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Previous biochemical studies have shown that both hypoxia and hypercapnia can markedly affect brain monoamine metabolism. The physiological significance of these changes is, however, difficult to evaluate based on biochemical data. It has been suggested by Carlsson and coworkers that hypoxia causes activation of brain NE neurons. The brain NE neurons in the nucleus locus coeruleus (LC) seem to be involved in a variety of behavioural (wakefulness, anxiety, stress) and physiological (cardiovascular) functions. Thus, we have utilized single unit recording techniques to study directly these brain NE neurons under hypoxia, hypercapnia as well as 100% O_2 in the inspired air. In the same experiments peripheral sympathetic nerve activity was registered in order to clarify the possible association between central and peripheral NE neurons.

Hypercapnia (3-15% CO_2 in the inspired gas mixture) caused marked simultaneous increase in both LC neuronal firing rate and sympathetic nerve activity. During hypoxia (about 10% O_2 in the inspired gas mixture) the LC neuronal firing rate was increased while the sympathetic nerve activity was decreased. When the pO_2-level was increased by letting the rat inspire 100% O_2 the LC neuronal firing rate was markedly reduced, while the sympathetic activity, if anything, was increased.

It is concluded that alterations in the availability of oxygen can markedly affect not only brain monoamine metabolism but also the physiological activity of e.g. the NE neurons. The data indicate that whereas in several situations central and peripheral NE neuronal activity is simultaneously and qualitatively similarly affected, this does not appear to be a general principle.

This work was supported by the Swedish Medical Research Council (projects nos 4747, 4764 and 00016)

D 5

A longla ting afterhyperpolarization following single spikes in CA1 pyramidal cells in the hippocampus

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Dept of Physiology Uni ersity f Göteborg Sweden

In most central neurones studied the soma-dendritic spike is followed by a longlasting afterhyperpolarization (AHP) This AHP is caused by a calcium activated permeability increase to potassium ions and is a major intrinsic factor for firing control In contrast to most other cells hippocampal pyramidal cells are believed to lack this AHP the spike being followed only by a longlasting afterdepolarization (ADP) (Kandel & Spencer 1961) However it was recently shown that longlasting repetiti e activation f CA1 pyramidal neurones was followed by a longlasting AHP (0 5 1 sec) probably caused by a calcium-activated potassium conductance (Hotson Schwartzkroin & Prince 1977) The questio then arising is whether this AHP can also be detected following single or short train of spikes
In the present experiments (performed on transverse slices of guinea pig hippocampus) CA1 pyramidal neurones showed an AHP following a single or a short train (2 5) of spikes Thi AHP which followed the ADP generally consisted of two phases an initial one of shorter duration (200-350 msec) reaching a peak amplitude 80-125 msec following spike onset and a later one of long duration (2 5 sec) reaching a peak amplitude some 500 msec following spike onset Following a single spike the AHP was usually dominated by its initial phase (peak amplitude around 1 mV) the later phase could be absent Howe e with repetitive activation the later component grew generally more than the initial one and the AHP could be dominated by its later phase The AHP was associated with a change in conductance the time cou of which ran parallel to that of the AHP Thus when th AHP was dominated by its later phase the conductanc change increased slowly with time reaching a maxim value some 500 msec following spike onset Followin the peak of the AHP the conductance change showed an exponential decay The AHP amplitude was potential-dependent decreasing and reversing in polarity with h perpolarization of the membrane The reversal was however asymmetrical the initial phase reversing a a more hyperpolarized level
Thus as in other central neurones the spike in CA1 pyramidal neurones is followed by an AHP presumabl caused by a potassium conductance increase Compar to other neurones this AHP is unusually longlasting w a duration up to 5 sec which is 50-100 times longer t that normally f und Some findings indicate that the A can be composed of two somewhat different ionic processes

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D 6

Chang th t f amyl se ec tion d ng p l g d t mul t on

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In xpe ime t on li ry amyl ecretion it i gene lly umed that th magnitud f the secret ry pon i d pend t on the amou t f p ef rmed amyl se i the gland T bt i unif rm exp iment l condition the nimal ually t rved f 24 hou ; t that time b t 90% f th amyl i found i the ec t ry gran l f tion (P tten Will ams & Cope 1977)
Stimul tions xp iment then ft carried out f ve l hou b t t i t known if he t f myl sec tion d g conti ou depl tion i ff t d by th de d amount f amyl i the gl nd it i known if only the pref rmed amyl ava l bl f se tion
In bbit undo than the l th light pa tid duct wa ca l t d t ll t liva l 5 mi i t rval l expe iment l ting 2 hou The amyl se s tion vok d by sympathati ti mul tion at 1 Hz wa rel ed l li ry flow (60-80 1/mi) p oduced by p ympatheti rve timul tion t 1 5 2 H (Gjö trup 1979)
The timul tion were continuous th oughout the experiment
Th res lt indi t th t d i g ch fi t 15 20 mi f timul tion the amyl se once t tion i about 3000 unit / 1 but ft 60 mi it h d c e ed t 25 50% lthough at l ast 50% f the gland lar content f amyl remained An the 60 mi of timul tion f rther de sed the s li ry con t tion t bout 10% f i iti l value
In th xperime t abbit p otid lob l were incub ted i g d Kr bs-Ri ge medium f 2 ho and timul t d with i op en li 10^{-6} M d ri g th first hou (Asking & Gjö trup 1980) Th ti sue we he hed and rei cubated f anothe hou with Kr b R nger upplemented ith amino id d g t Eagl MEM
At the end f thi p iod th amount f amyl emai ng i the t uo nd that rel d i t b th wa say d t obt i the t t l amount In fou xpe ime t the tot l amount f amylas i un timul ted ontrol and in th timul ted ti ue found t be th ame angei g between 1300-1500 un t /100mg ti ue I control 90% f the amount wa found the ti uo whil 50% wa l ft l the t mul ted ti ue
Th pe me t suggest that lthough l rge amount f amyl may em av l bl f cre ion the t f amylas l ca not be mai t ined but d w th inc i g depl ti f amyl se Th re lt lso gge t that only p f rmed amyl i ted which i support d by th l k f mylase f rmation i the i it pe ime t

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Br-ergocryptine (CB-154) inhibits prolactin (PRL) and growth hormone (GH) production in cultured rat pituitary tumour cells

F JOHANSEN K.M. GAUTVIK, HAUG, SAND O CLAUSEN

Institute of Physiology University of Oslo Norway Department of Phys logy Norwegian College of Veterinary Medicine Os and Institute of Patho ogy University of Oslo Rikshospitalet Oslo Norway

Rat pituitary tumour cells (GH_3 cells) which synthesize and secrete PRL and GH in vitro react to several physiologically occurring hormones as thyroliberin (TRH) and 17β-estradiol in manner analogous to normal pituitary cells. Shorttern dopamine (DA) treatment inhibits PRL secretion in these cells (1) We have studied the ffects of the ergot derivatives CB-154, which acts as potent dopamine receptor agonist in the anterior p tuitary gland (2) and the binding ^{3}H-dihydroergocryptine to GH_3 cells at 37°C.

Low concentrations of Br-ergocryptine ($5x10^{-6}$M) inhibi PRL secretion by about 30% after 30 min and synthesis by about 60% after 3 days f treatment. GH secretion and synthesis as well as cell growth were not ecifically affected this concentration. Howeve raising the concentration of CB-154 (above 10^{-5}M) an increasing inhibition of PRL synthesis (more than 90) was observed in addition to reduction in GH production and cell proliferation These effects were reversible on cessation f CB-154 treatment Intracellular electrophysiological registrations showed inhibition f spontaneously occurring action potentials in the GH_3 cell

After 6 days of treatment the cell proliferation was reduced about 80% in comparison with controls Microflow cytofluorometry demonstrated an inhibition in cell cycl traverse in the $_1$ phase of growth.

Binding f ^{3}H-dihydroergocryptine was rapid saturable and partly reversible 37°C

In conclusion CB-154 has an ffect analogous to DA in GH_3 cells by inhibiting PRL and GH secretion and synthesis in dose and time dependent manner The short time ffects occur rapidly and cause changes in cell membrane characteristics and hormone secretion whil longer treatment periods cause inhibition also of hormone synthesis and cell growth

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D 8

Capillary supp and cross sectional area of low and fast twitch ibres in skel tal muscle from males and females.

INGJER C SALTIN

August Krogh Institute, Uni ersity of Copenhagen Denmark

Human skeletal musc the degree of cap llarization of each of the major fibre types is difficult to evaluate separately as the low and fast twitch ibre are heterogeneous mixed and capillaries are shared between different fibre One approach to the problem is to analyze muscles with large dif erences in fibre composition

elve healthy young subjects (6 men and 6 women, moderately to well trained) had muscle biopsies taken from tricep brachii (TB) vastus lateralis (VL) and soleus ( ) ibre lassif cation in slow twitch (ST) and fast twi ch (FT) ibres was based on histochemical staining for myofibrillar ATP-ase after preincubation at pH 4 3 and 10 3 Capillaries were histochemically visualized with the amylase-PAS method Cross sectional fibre area and the lesse fibre diameter were determined on photographs of the amylase-PAS stain

The fibr compositions were for the 39% ST for VL 61% ST and for S: 71 ST with no iff rences between the sexe The number of capillaries around (CA) ST fibres was less in TB compared to VL or for men and women (Table)(p 0 05) For the FT fibres CA was less in TB than in (0 05) Part of the explanation for the variation in CA was the difference in cross sectional area of the fibr (FA) (Table) For FT fibres men had larger FA in TB and VL but not in S (0 05) Comparing ST fibres between the three muscles the FA was smallest in TB compared to VL or for men and women (p 0 05) with similar trend for FT fibre To evaluate capillary density for the two fibre types the variable fibr type area per capillary" (AC μm^2) was calculated This variable was igni icantly different between ST and FT fibres in all the muscles for the men and in TB for the women

Tabl I. Mean values for capillaries around (CA) slow and fast twitch (ST,FT) fibres their cross sectional area (FA) supplied by each capillary AC) in triceps brachi (TB) vastus lateralis (VL) and soleus (S)

		TB ♂	TB ♀	VL ♂	VL ♀	♂	♀
CA (no)	ST	9	3 9		4 6	6 9	7 8
	FT	5 1	3 7	6 2	4 0	6 6	8
FA (μm^2 x 10^3)	ST	3 9	2 9	2	5 0	6	7 8
	FT	5 8	3 9	6	4 4	8 8	7 9
AC (μm^2 10^3)	ST	0 8	0 8	0 8	1 0	0 9	1
	FT	1 1	1 0	0	1	1 3	0

Several conclus ons are possibl First the there are differences in the areas supplied by each cap llary when comparing ST and FT fibres Second that the differences which exist in CA between the muscles are likely related to the size f the fibres and third the there are onl minor sex differences in those variables measured.

D 5

A longlasting afterhyperpolarization following single spikes in CA1 pyramidal cells in the hippocampus

B GUSTAFSSON & H WIGSTRÖM
Dept of Physiology Uni ersity of Göteborg Sweden

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D 6

Change i th t f amyl se sec tion d ing p longed timul t on

B. ASKING P GJÖRSTRUP & H PÄRSSON
I tit t f Physi l gy Un versity f Lund Swed

I xp iment on sali ry amyl ecretion it i gen lly sumed tha th magn tud f he ec ret ry spons i depend t on the amount f p f rmed amylase the gland T obta unif rm xpe iment l ondition the mal su lly t rved f 4 hou i t that time bout 90% f the amyl i found the ec t ry gran l f tion (Pr tten Williams & Cop 1977) Stimul tions xp ime t th ft carr ed out f ve l hou but t i ot known if the t f amylas secretion d g cont nuou depl tion i ff t d by the d sed amount f amyl i the gl nd so it i known if only the pref rmed amyl se il bl f ec t on In r bbit unde than ane the i th right pa otid duct was canul ed t ll t liva i 5 mi i t rv l i expe me t l ting 2 hou The amyl se ec tion voked by ymp theti ti mul tion t l Hz wa l ased in li ry flow (60-80 l/mi) produced by pa sympatheti rve timul tion t l 5 2 H (Gjö trup 1979) The timul tion we conti uou throughout the xpe iment The re lt indicat that d ing th fi t 15 20 mi f timul tion the amyl conce t tion i about 3000 unit / l but ft 60 mi it ha dere sed t 25 50% lthough t l ast 50% f the gl nd l cont t f amylas remained An the 60 mi f timul tion furthe dec sed the ali ry con ent tion t bout 10% f i iti l value In th xperime t abbit parotid lobules were incub ed i g sed Kr bs-Ri ge medium f 2 hou and timul ted with isop en line 10 M d i g the first hou (Asking & Gjörstrup 1980) Th sue we he ve hed and reincubated for an the hou with Kr b Ringe upplemented with amino id cc d g t Eagl MEM At the end f thi pe iod the amount f amyl se emai ing th ti ue od that rel as d int b th say d t obt i the t t l amount I fou xpe ime t the tot l amount f amylas i un timul ted control and i th timul ted tissue w found be the same gel g between 1300-1500 unit /100mg ti I control 90% f the amount wa found i the ti ue whil 50% wa l ft the imul ted ti sue Th expe men sugge t that lthough l ge amoun f amyl may rem av il bl f se ion the t f amylas l not be mai ined by de se with inc l g depl tion f amylase The re lt lso sugge t that only p f rmed amyl ec ted whi h i supported by th l h mylas f rmation i he i lt xpe iment

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D 7

r-ergocryptine (CB-154) inhibits prolactin (PRL) and growth hormone (GH) production in cultured rat pituitary tumour cell

/ E. JOHANSEN K.M. GAUTVIK, E HAUG O SAND A.J CLAUSEN

Institute of Physiology University of Oslo Krogh Department of Physiology Norwegian College of Veterinary Medicine Oslo and Institute of Pathology University of Oslo Rikshospitalet, Oslo Norway

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Binding of ^{3}H-dihydroergocryptine was rapid saturable and partly reversible at 37°C.
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D 8

Capillary supply and cross sectional area of slow and fast twitch fibres in skeletal muscles from males and females

INGJER F SALTIN

August Krogh Institute University of Copenhagen Denmark.

In human skeletal muscle the degree of capillarization of each of the major fibre types is difficult to evaluate separately as the slow and fast twitch fibres are heterogeneously mixed and capillaries are shared between different fibres. One approach to the problem is to analyse muscles with large differences in fibre composition.
Twelve healthy young subjects (6 men and 6 women, moderately to well trained) had muscle biopsies taken from triceps brachii (TB) vastus lateralis (VL) and soleus (S). Fibre classification into slow twitch (ST) and fast twitch (FT) fibres was based on histochemical staining for myofibrillar ATP-ase after preincubation at pH 3 and 0 3. Capillaries were histochemically visualized with the amylase-PAS method. Cross sectional fibre area and the lesser fibre diameter were determined on photographs of the amylase-PAS stain.
The fibre compositions were for TB 39% ST for VL 60% ST and for S 73% ST with no differences between the sexes. The number of capillaries around (CA) ST fibres was less in TB compared to VL or S for men and women (Table)(p 0 05). For the FT fibres CA was less in TB than in S (p 0 05). Part of the explanation for the variation in CA was the difference in cross sectional area of the fibres (FA) (Table). For FT fibres men had larger FA in TB and VL but not in S (p 0 05). Comparing ST fibres between the three muscles the FA was smallest in TB compared to VL or S for men and women (p 0 05) with a similar trend for FT fibres. To evaluate capillary density for the two fibre types the variable fibre type area per capillary" (AC μm^2) was calculated. This variable was significantly different between ST and FT fibres in all the muscles for the men and in TB for the women.

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Several conclusions are possible. First that there are differences in the areas supplied by each capillary when comparing ST and FT fibres. Second that the differences which exist in CA between the muscles are likely related to the size of the fibres and third that there are only minor sex differences in those variables measured.

D 5

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In most central neurones studied the soma-dendritic spike is followed by a longlasting afterhyperpolarization (AHP) This AHP is caused by a calcium-activated permeability increase to potassium ions and is a major intrinsic factor for firing control In contrast to most other cells hippocampal pyramidal cells are believed to lack this AHP the spike being followed only by a longlasting afterdepolarization (ADP) (Kandel & Spencer 1961) Howev r it was recently shown that longlasting repetitive activation f CA1 pyramidal neurones was followed by a longlasting AHP (0 5–1 sec) probably caused by a calcium-activated potassium conductance (Hotso Schwartzkroin & Prince 1977) The question then arising is whether this AHP can also be detected following single or short train of spikes

In the present experiments (performed on transverse slices of guinea pig hippocampus) CA1 pyramidal neurones showed an AHP following a single or a short train (2–5) of spikes This AHP which followed the ADP generally consisted of two phases an initial one of shorter duration (200–250 msec) reaching a peak amplitude 50–125 msec f llowing spike onset and a later one of long duration (2 5 sec) reaching a peak amplitude some 500 msec following spike onset Following single spik the AHP was usually dominated by its initial phase (peak amplitude around 1 mV) the later phase could be absent Howev r with repetitive activation, the later component grew generally more than the initial one and the AHP could be dominated by its later phase The AHP was associated with a change in conductance the time course of which ran parallel to that of the AHP Thus when the AHP was dominated by its later phase the conductance change increased slowly with time reaching a maximum value some 500 msec following spike onset Following the peak of the AHP the conductance change showed an exponential decay The AHP amplitude was potential dependent, decreasing and reversing in polarity with hyperpolarization of the membrane The reversal was however asymmetrical the initial phase reversing at a more hyperpolarized level

Thus as in other central neurones the spike in CA1 pyramidal neurones is followed by an AHP presumably caused by a potassium conductance increase Compared to other neurones this AHP is unusually longlasting with a duration up to 5 sec which is 50–100 times longer than that normally found Some findings indicate that the AHP can be composed of two somewhat different ionic processes

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D 6

Change in th t f myl se tion d ing pr longed timul tion

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In expe iment on sali ry amylase ecretion it i gene lly sumed th t th magnitud f the ec et ry pon i d pendent on the amount f preformed amyl se i the gland T obt i unif rm exp iment l condition th imal ually t rved f 24 hour ; t that time about 90% f the amyl i f und i the ec t ry g an l fra tion (P tten Williams & Cope 1977)
Stimul t on xpe iment the ft carried out f ve 1 hours b t it i t known if th at f amylas ec tion d ing continuou depl tion i ffect d by th d reased amount f amyla i th gl nd it i known f only th pref rmed amylase i avail bl fo sec t on
In r bbit unde urethan anesthe i th right pa tid d t w ca l ted t ll t l va i 5 ml i t rv l i exp riment l ti g 2 hou The amyl se ec tion voked by ymp theti t mul tion t 1 Hz wa r l ased i livary flow (60–80 1/ml) produced by p ra ympatheti rve timul tion t 1 5 2 H (Gjö trup 1979) The timul tion were continuous th oughout the xpe iment
Th re lt indicat that d ri g the fi t 15 20 ml f timul tion the amyl se once t tion i about 3000 unit /ml but aft r 60 mi it ha de c ased t 25 50% lthough t l ast 50% f the gland lar content f amylas remained Anothe 60 mi f timul tion f rthe de sed the li ry con t t on t bout 10% f i itial value
I th expe ime t abbit p tid lobules were incub ted i g sed Kr bs-Ringe medium f 2 ho and timul d with i op en li 10 M d i g th f t hou (Asking & Gjö strup 1980) The ti we he wa hed and rei ubated for an the hou with Kr bs-Ringe supplemented ith amino id cordi g t Eagl MEM
At th end f hi pe iod th amount f amyl s emai ing i th ti sue and that rel ased i t b th wa say d t bt i he t l amount I fou xpe ime t the t t l amount f amylas i un timul t d ontrol and i th timul ted ti ue v found be the same rangei g b tween 1300–1500 un t /100mg i ue In control 90% f th amount wa found i the ti ue wh l 50% wa l ft i the t mul ted ti ue
Th expe me sugge tha lthough l rge amount f amyl se may emain l bl f se t on the t f amyl j not be mai t ined be decr se v th i g depl tion f amyl se Th re it lso gge t th t only pref rmed amy l i ecre d which i suppo t d by he lack f mylas f rmation he i vit pe iment

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D 11

Effect of exercise on lecithin-cholesterol acyltransferase (LCAT) ac y I men

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Lecith n-cholesterol acyltransf ase (LCAT) has central rol i lipoprotei metabolism Its mai function i to produce chol terol esters i high density lipoprotei (HDL) parti les (eg Glomset 1970) There i lso evidence that LCAT may have role i the removal f cholesterol from i ues However very little i known about the regula-tion of the enzyme acti i y i the presen tudy the effect of tr i ing on serum LCAT acti ity i reported

A group of 20 men trained unde control f 15 weeks at leas three hours week The i ing consisted mai ly f running or skiing During the same per od control group f seven men retained the normal physical acti ity A the beginning of the period the groups did no diff i rel tion to rel tive body weigh age or physical fitness The effect f i ing on plasma lipids and lipolyti enzymes these subjects have been reported ea li (Peltonen l 1980) Serum LCAT acti ity was measured dur ng the trai ing period i 19 trai ing and i con rol ubject according to Al indo l (1978)

Trai ing caused ignifican (P<0 001) in-crease i physical fitne while no change occurred i control i the t i ing group LCAT acti ity inc eased from 13 9+2 2 nmol/ l/h to 31 1 3 2 nmol/ l/h (P<0 001 paired t-tes) whereas i control the acti ities emained the same Bef re but not fter the t i ing LCAT correl ted positively ith serum t iglyce ides (P<0 05) and ith posthepa i plasma hepati lipase (P<0 10) i the combined groups

The present res lt ugges an increasing f fect of i ing on serum LCAT i ity Whethe thi i direct influence f t i ing on the enzyme i not lear Apoprotei A I which i an tivato f LCAT i lso increased i physical ly active ubjects (Lehtonen l 1979) and thi could be one pos ibl explanation for the observed change Togethe ith the increased HDL cholesterol conten ion (Peltonen t l 1980) thi levated enzyme ti ity could have ben-fi i l ffect on chol terol removal from ti ues

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D 12

Exercise induced changes i human serum f ty acid compos on

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The f tty ac d composi ion of l pids i the blood and d fferent i sues ha recen ly gained growing attention i health surveys especi lly i the search of new isk factors f therosci rosi (eg. Dyerberg l 1975 Sanders l 1978) The rol of physical exercise has generally not been taken to account as possibl effector f human fatty ac d composi ion i the presen tudy we have analyzed the serum total f tty acid compo-sition du ing controlled physical trai ing program.

The dura ion of the trai ing program (Peltonen et l 1980) was 15 weeks consi ing mai ly f running or skiing unde control leas three hour week The ubjects (20) and the control (7) were previously un rained healthy men

The total lipid frac ion of the fas ing serum samples was isol ted and purified essen i lly ac-cording to Folch l (1957) The f ty acids were analyzed as thei methyles (van Wijn-gaarden 1967) by capill ry gas chromatography using butanedi l succinate as the liquid phase and flame ioniza ion detec ion

During the tes period there appeared to be tendency to reduced level f polyunsaturated long-chained f ty acids i serum A ignifican de-crease was found i the concen ion f 22 5ω3 (P<0 05) and 22 6ω3 (P<0 02) acids as compared to the con rol i 20 4ω6 and 20 5ω3 the small reduc ion (10-20 %) wa no howeve tati tically ig ifican When calcul ted as combined group the long-chained (18 C toms) polyuns tur ted f tty acids di i ished ignifican ly (about 10 % P<0 05) du ing trai ing The i ω6 ids/ω3 acids in-creased ignificantly i the rai ing group (P<0 025) as calcul ted by the paired t test whereas no change occurred i the con rol group Linol i acid (18 2ω6)/ achidoni acid (20 4ω6) ra i lso increased i the trai ing objects (P<0 10) whereas i the control the mean f thi ra i had decreased t the same ime i the los saturated/unsaturated or saturated/18 1ω9 18 2ω6 no ignifican changes we obtained during t in-ing

At presen i i difficult to say whether these exercise induced changes have any importance i rel tion to health eg development f theros le-rosi F tty acid analyses of pecifi lipid lasses re needed before thi problem can be i ified

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D 9

The Effect f 3 5 -cycli Adeno ine Monophosphate (cAMP) on Glucose Transport in Adipose Tissu and Soleu Muscl f the Rat

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Insulin has been shown to induce decrease in th cAMP content f its targ t ll but littl i known about the ignifi ance f thi ffect f r the action f the hormon on glucose transport

The p esent study wa undertaken with the pur pose of assessing the possibl rol f cAMP a second messenger for the acti tion f the glucose transport system

I hol epididymal f t pads cAMP (2mM) and insulin (100μU/ml) timul ted the conv rsion f U-^{14}C labelled D-glucose into CO_2 and triglycerides by 30% and 48% especti ely When added tog ther t the same concentrations an increase f 180% was obtained Adrenaline (1 $^{-5}$M) stimulated glucose metabolism by 117% in the absence and by 14% in the presence f insulin (100μU/ml)

Glucose transport was asse sed by measuring the efflux of ^{14}C 3-0-methylglucose (^{14}C 3 0-MG) from pr loaded tissue (Clausen 1969) In whol epididymal f t pads both cAMP and insulin stimul ted ^{14}C 3 0-MG efflux and the effects wer additive (Tabl 1)

Adrenaline and salbutamol which stimul t adenylate cyclase both induced stimulation of ^{14}C 3-0-MG efflux Cytochalasi B abolished the ffect f all these stimuli indicating that it affects activation f carrier-medi ted ^{14}C 3 0-MG transport

In soleus muscl cAMP (2mM) did not i terfer with the effect of insulin (1mU/ml) on ^{14}C 3-0-MG fflux Adrenaline (10 $^{-5}$M) and salbutamol (10 $^{-5}$M) timulated ^{14}C 3-0-MG efflux by 68% and 50% espectively

Thes data argue against the idea that decrease i the cytoplasmi level f cAMP mediates the action f insulin on glucose transport in its major targ t tissues

Tabl 1 The effects of cAMP on ^{14}C 3-0-MG efflux from epididymal fat pads

	Fractional loss f ^{14}C 3 0-MG/min ( )		
Control	0 013 ± 0 006	(8)	
Adrenaline (10^{-5}M)	0 020 ± 0 001	(3)	p<0 001
Adrenaline (10 $^{-5}$M) insulin (25μU/ml)	0 023 ± 0 002	(4)	p<0 001
Salbutamol (10^{-5}M)	0 020 ± 0 004	(4)	p<0 025
cAMP (0 1mM)	0 015 ± 0 001	(4)	p<0 2
cAMP (0 5mM)	0 020 ± 0 001	(4)	p<0 001
cAMP (1 0mM)	0 018 0 001	(5)	p<0 005
cAMP (2 0mM)	0 017 0 001	(10)	p<0 001
Insulin (25μU/ml) cAMP (2 0mM)	0 029 ± 0 002	(3)	p<0 001
			p<0 05
Insulin (25μU/ml)	0 021 ± 0 001	(3)	

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D 10

The Rol f Ca^{++} Ions in the Activ tion f the Glucose Transport System in Muscl and Adipose Tissu

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A wid variety of agents mimick the action f insulin in timulating glucose transport The pur pose f the present study ha been to examine the ignificance f cal ium in the mechanism of action f these compounds

All expe iments wer performed ing intact soleus muscl or whol epididymal f t pads f the t The function f the glucose transport system wa assessed by measuring the fractional los f ^{14}C or ^{3}H-labelled 3-0-methylglucose (3 0-MG) from preloaded ti sues The fractional l ss f ^{45}Ca wa sed as an indicator f r the cytoplasmi Ca^{++} level (Clausen Elbrink & Dahl Hansen 1975)

12 different agents whi h wer found to produce from 2 to 6 f ld stimulation f cytochal in B suppr ssibl 3-0-MG fflux all induced highly ignificant (p<0 001) increase in the fractional los f ^{45}Ca both in muscles and f t pads In each instance the ise in the ^{45}Ca lease could be detected befor or imultaneously with the ti mulation f 3-0-MG efflux

Preloading with combination f ^{45}Ca and ^{3}H 3 0-MG allowed direct comparison between the changes in the fractional loss f the two isotopes from individual muscl When t ted t 4 different concentrations the 5 age ts listed in Fig 1 induced dose-dependent increase in the fractional los f ^{45}Ca- and ^{3}H-activity Regression analysis showed significant positive correlation between thes two parameter

The esults support the idea that in the major targets for insulin action Ca^{++} ions participat in th mechanism f activation f the glucose transport system

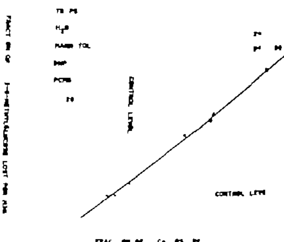


Fig 1 Effect f gl cose transport timuli on the efflux (f actional loss) f ^{45}Ca and ^{3}H 30-MG from soleu muscles Each point epre ents mean f 3 observ tions

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The influence of autonomic agents and hypoxia on gas exchange in perfused fish gill

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Blood enters the complex gill v cul ture through filament arteries di tributing into secondary lamellae where gas exchange occurs Leaving the lamellae blood proceeds i to the arteri l systemic circulation via efferent filament and branchial arteries Alternatively post lamella blood may pass nutritive vessels of the gill branchial arteries or proceed through anastomosis to the internal jugular vei

Earlier studies have demonstrated that catecholamines favour flow i the arterio- rteri l pathway causing reduced -v flow Such a flow shift is l kely to raise the O_2 transport effi ciency of the gills at times of increased sympathetic activity The objective f this tudy was to assess if adrenaline and acetylcholine (Ach) affect the oxygen level of the perfusate in perfused gill preparation Additionally the effects of amb ent and perfusate O_2 tensions on the gill vascul ture resistance have been studied

The gill apparatus of the cod Gadus morhua was perfused i itu via the ventral aorta The O_2 tension of the perfusion fluid and amb ent water was regulated th gas mixing pumps Arterial and venous outflows and thei O_2 tensions were continuously monitored as was ventral aortic perfusion pressure.

Adrenaline (10⁻⁶M) increased both volume nd pO_2 of the arteri l outflow by 40% and 45% res pectively while concurrently the venous effluent pO_2 and outflow declined Ventral aorta pressure fell indicating reduced gill vascula resistance following adrenaline adm istration

Ach ($3x10^{-7}M$) caused only very small changes i the flow f both effluents but a drop i both arteri l nd venous pO_2 The increased pO_2 of the arteri l outflow associated with adrenaline injection is likely related to microcirculatory re-arrangement invol ing lamell recruitement The increased gill culature resistance with Ach administration may result from pillar cell contractions levating lamell resistance

Hypoxic conditions induced by lowering perfus ion fluid pO_2 or ambient pO_2 resulted i levated ventral aort c pressure The site of the increased resi tance to hypoxia is probably the same that for Ach ince this agent gave only a slight addi tional increase i branchi l resi tance compared to the response i a non-hypoxic ituation

Cerebral blood flow and ^{14}C-deoxyglucose accumulation in the brain

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In the CNS the metabolism is correlated to the activity both temporally and spatially The blood flow is unde strong influence of local factors and as consequence one can expect that under normal condit ons there is good correlation between local acti ty and local blood flow Good evidence for such correlation has been presented e.g. by Ingvar and Lassen (1977)

In experimental animals local blood flow can be measured with the microsphere method.

Local cerebral glucose consumption can also be expected to reflect local acti ty in the CNS glucose being the main nutrient This presumes that oxidative metabolism dominates by far regions with anaerobic glycolysis might have low metabolism and still high glucose consumption.

A method for studies on local glucose consumption was devised by Sokoloff (1975 1977) the main assumption being that accumulation of deoxyglucose which enters the cells very much like glucose and is then phosphorylated but not metabolized any further reflects glucose consumption.

We have invest gated the relation between regional blood flow as measured with icrospheres and deoxyglucose accumulation in 9 regions in one anesthetized rabb t and 17 regions in one monkey under pentobarbital anesthesia After the preparation of narrow fraction f 9 um microspheres these were injected into the l ft heart vent icle about 40 in fte the injection f ^{14}C-deoxyglucose the animal was killed and the brain frozen and sect oned The deoxyglucose concentrat on in sections was det rmined using densitometry f autoradiographs The number f spheres was determined in the same regions by counting in icroscope

In both animals there was four to five-fold variat on in deoxyglucose concentration from parietal white matter to the gray matter of the inferior colliculus The variation in sphere concentration was about 20-fold The relationship between concentration f deoxyglucose CD and the concentration of spheres CS appeared nonlinear and seemed to be best fitted by an equation f the form CD=k √CS Both methods thus seem t give measures reflecting local metabolism but the coupling between local blood flow as measured with 9 um spheres and deoxyglucose accumulation probably varies from one part of the brain to another

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D 13

Brain uptake of mannitol and sucrose after cerebral ischemia in normo- and hyperglycemic rats

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The permeability of the cerebral capillary endothelium is not affected by a short lasting ischemic episode from which the animals recover completely However pretreatment of such animals with glucose causes them to succumbe compeletely after ten minutes of global ischemia (Siemkowicz &Hansen 1978) The absent restitution may be a consequence of increased blood-brain barrier permeability affecting ion and neurotransmitter homeostasis In order to investigate this possibility the permeability of the capillary endothelium to mannitol and sucrose was studied after ten minutes of global cerebral ischemia Normo- or hyperglycemic rats were strangled by inflation of a pneumatic cuff and reduction of arterial blood pressure to 50 mmHg Plasma glucose was changed prior to ischemia by i p glucose administration

The regional permeability-surface area product (PS) was measured after i v bolus injection of 14 C-mannitol and 3 H sucrose by the integral m thod

The PS for mannitol averaged 0 17$\pm$ 0 02 (sd)ml/(100g) min in normoglycemic rats before ischemia and the mannitol sucrose ratio averaged 2 3$\pm$ 0 5

When plasma glucose was raised to 28 mM PS for mannitol decreased to 0 09$\pm$ 0 02 and the mannitol:sucrose ratio remained unchanged (2 6$\pm$ 0 1)

Immediately after ischemia PS for mannitol increased 2 3 fold above the respective control values but two hrs after ischemia PS was again reduced to 80% of control values in the normoglycemic rats and slightly above control values in the hyperglycemic rats The mannitol:sucrose ratios were never significantly different from the respective control values

The results indicate that the permeability of the cerebral capillary endothelium to small polar solutes is unchanged after ten minutes ischemia It is therefore unlikely that the detrimental effect of hyperglycemia on the outcome of cerebral ischemia is a consequence of decreased integrity of blood brain barrier

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D 14

Inotropic eff ct of salbutamol and pren alterol on pig myocardium in vitro

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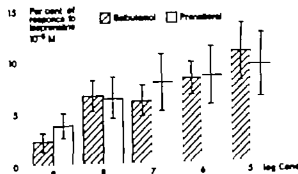
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Salbutamol is a β_2- drenocepto timula tor (Cullum et al 1969) used clinically in the treatment of asthma and prenalterol (like the racemic H 80/62) i a β stimulator with marked positiv inotropic action on the heart (Carl son et al 1977 & Johnsson et al 1978)

A series of experiments was planned to classify the β adrenoceptor in different tissue from pigs using those selective adren rgic agoni ts

Contractile force was recorded in electrically stimulated strips or trabeculae of porcine myocardium mounted in vitro in standard Krebs solution

Salbutamol in concentrations up to 10 ^{5}M excerted a expected only a weak inotropic influence; the maximum increase in active force was 11±4% (m an±SE) of the r ponse to isoprenaline 10 ^{5}M Howev r also prenalterol in dose up to 10 ^{5}M had a weak a tion 10±3% relative to i oprenalin Thi low intrinsic ffic cy of pren lterol in thes experime ts contr sts with its documented action in vivo and may be due to pecie difference or to the unphysiological conditions in vitro The pre ent in vitro tudy th refore does not con lusiv ly cla ify the porcine myocardial β adr noceptor i to β or β_2 predominance



R f rence

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D 19

Retinal and brain uptake of EDTA sodium and
[illegible] in vivo.

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The blood-retinal barrier (BRB) is considered an analog to the blood-brain barrier (BBB) It is known that small water-solubl substances such as sodium-fluorescein (Grayson & Laties 1971) and horseradish peroxidase (Smith & Rudt 1975) and sodium (Törnquist 1979 Tornquist Alm & Bill 1979) do not easily pass the BRB but the permeability characteristics of the BRB are less well known than those of the BBB. The present tudy was undertaken to compare the penetration into and through the BRB and the BBB by determining the concentrations of various substances in retina and brain fter an or an intracarotid injection.

The extravascular concentrations f 51-Cr-EDTA were determined in retina and brain 60-90 fter i.v. injection in albino rats In these expts 125-I-albumin was included as an intravascular marker and the activities due to 125-I and 51-Cr in weighed blood samples were used to calculate and subtract the activity due to 51-Cr-EDTA located in the intravascular space By comparing the extravascular concentrations f 51-Cr-EDTA ratio between the permeability surface area (PS) products for retina and brain was obtained. This ratio was about 8 T determine whether this was due to higher permeability or larger surface area of the BRB the rate constants for the wash-out f freely diffusibl substance from retina and brain was determined This was done by determining the fraction f injected 3-HOH recovered in weighed samples f retina and brain at various times fte an intracarotid injection as bolus The rate constant for the BRB was about 7 times that for the BBB indicating that larger surface area i the main factor determining the difference in PS-products between the BRB and the BBB.

In third series f expts. the concentrations f 22-Na and 125-I in retina and brain 5-7 fter an intracarotid injection were compared. The retinal concentrations were 5-9 times that in the brain indicating that also ions enter the retina more easily than the brain. The concentrations f 22-Na and 125-I in the brain were the same while in the retina the ratio Na/I was 1.4 Transport mechanisms or lectrical forces may explain this difference in retinal uptake of Na and I

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D 20

Hexose transport across the blood-retinal barrier

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In analogy to the blood-brain barrier (BBB) there is also blood-retinal barrier (BRB) tuated in the retinal capillary wall and the retinal pigment epithelium. Metabolic substrates are transported across the BBB by carriers exhibiting saturability and stereo-specificity The im f the present study was to compare the passage f hexoses through these two barriers.

The brain uptake index(BUI)method (Oldendorf 1971) was modified to obtain also the retinal uptake index (RUI) Thus RUI and BUI were determined for various monosaccharides alone and in the presence of 40 mM unlabeled D-glucose (cross-inhibition) RUI and BUI were also determined for D-glucose inhibited by unlabeled D-glucose in concentrations between 1 and 300 mM (self inhib tion)

The carrier with the same relative ffinity to several hexoses was found for both barriers The results obtained with self inhibition of D-glucose was used to calculate the K_m-values for the transport across the BRB and BBB. K_m-values calculated for self inhibition up to 5 mM were 13 7 and 5 5 mM for BRB and BBB respectively However, they increased gradually with increasing concentrations of unlabeled D-glucose above 5 mM. Two possible explanations are accelerative exchange diffusion and two transport systems It was concluded that accelerative exchange diffusion of glucose across the BRB and the BBB is the most likely explanation

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D 17

Effe ts f ubstan P,PGE$_2$ and apsai in on the pupill ry sphinct r modifi tion by t trodotoxin

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Th rabbit eye i very us f l in studies on inflammatory process es Two kind of mechanisms hav been particul ly tudied one involving the rel as of pro t glandins the the invol ing a noncholinergi neur l component

Prostaglandins in the eye p oduce mi sis a ise in intraocul pre ure and breakdown of the blood-aqueous b rri Neuronal irritation produced e g by antid omi timul tion of the trigeminal nerve o adminstration of irritant such as caps icin has simil r ffects The neuronal ffect seem to be due to release f ubstanc P imilar substanc (Bill t al 1979)

We have inve tigated the ffects f substanc P PGE$_2$ and cap ai in on the pupil iz and t what exte t the ffects requir normal nerv function I tracame l i jection of 10 ng t trodot i (TTX) on one id was used t block nerve conduction i that eye At thi dose antidromi stimul tion f the trigeminal rv had p actically no ff t on the pupil iz indi ting fficie t blockad

The dose-re ponse r l tionship f i tr mee l substance P w not signifi antl aff ted by TTX, slight contraction f the sphinct pupillae occurri g t 200-400 pg and ma ked miosis at 1000-2000 pg

PGE$_2$ doses of 15-40 ng produced moderat to marked miosi on the control side but did not have distinct ffects on the pupil size on the TTX ide

Capsaicin 30-120 ng was strongly mioti on both sides

Butl and Hammond (1977) demonst ated that (t trigeminal denerv tion f the eye PGE$_2$ and caps in have no mi ti ffect But substance P is strongly mioti (Butle unpublished)

It s ems likely then that the miosi caused by s bstance P is due to direct ff t on the sphinc t pupillae muscl The PGE$_2$ miosi requi es neuronal impulse propagation and thus is not direct muscular effect PGE$_2$ miosis could be due t activation f prostaglandin recepto s on the peripher l nerve endings and ubsequent r l as f substanc P Capsaicin miosis require presenc of nerv s but an be produced even after blockade f nerv conduction s ggesting that thi substance cause release f substanc P r a imilar substanc by omo unspecific eff t on the permeabi lity of the nerve endings

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D 18

EFFECTS OF FACIAL NERVE STIMULATION ON OCULAR BLOOD FLOW AND THE INTRAOCULAR PRESSURE (IOP) IN THE CYNOMOLGUS MONKEY

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Morphol gical evidence f a pa a ympathetic nerv upply vi the faci l nerv t the oc l r and cer b l blood v ssel i well est bli hed I t acrani l stimul tion f the facial nerv i r bbits c uses vasodil tion in the eye and an increase i IOP Blood flow lso incr as in the ubmandibular gland The ff ts on the ye are si t t t choline gic and ganglioni blocked (Stj rns hant & Bill 1980) which indi t that the asodil tion is caused by non-conventional t ansmittor One possibl andid t i vasoactive i te tinal polypeptide (VIP) I travenous i jection f VIP i rabbits caus an incr ase i horoid l blood flow and IOP (Nil son & Bill 1979)

In the present tudy region l blood flow was det rmined in cynomolgus monkeys (n=7) usi g the labelled microsphe e method The faci l nerve on one ide was xposed sectioned and intracrani lly timulated t the i t rnal acousti po IOP was registr ted in both eye Three blood flow measurements were made in ach monkey one without timulation and two during timulation bef re and afte cholinergi (tropine) or ganglioni (hexamethonium) blockad

In 4 ther monkeys VIP was injected i and the ffects on the blood pressur and IOP we tudied

Cho idal blood flow on the timulated ide inc reased by bout 100% during stimulation IOP also inc ased on the timulated ide The ffects were re i tent t bolinergi blockade but were compl t ly abolished aft r ganglioni blockade Ce b al blood flow was not aff ted.During timul tion the blood flow i the submandibular gland increased 5 20 time Thi ffect was markedly educed ft holine gic blockade and aboli hed aft ganglioni blockade I i j tions f VIP aused a decreas i blood pres ure and an increas i IOP

The re lt indi t that faci l nerve timulation us vasodil tion i the eye vi ff t nerve that t l ast one ynapse i pre ent between the it f timulation and the pe ipher l nerve terminal and they ugge t th t VIP or some other unconventional t ansmitt i involved i the re pons

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D 23

Hyper- and hypoalgesic effects of 5-hydroxytryptamine (5-HT) agonist.

O.-G. BERGE, K. HOLE [illegible]

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Firing of ascending 5-HT neurons is depressed by stimulation of presynaptic receptors, e.g. by administration of low doses of the 5-HT agonist 5-methoxy-N,N-dimethyltryptamine (5-MeODMT; Trulson & Jacobs 1979) which has a preferential action on presynaptic 5-HT receptors (De Montigny & Aghajanian 1977). Higher doses of 5-MeODMT have postsynaptic agonistic effects as well (Fuxe et al 1972), depressing the response to radiant heat in the tail-flick (TF) test (Berge & Hole 1979). In the present study we investigated if stimulation of presynaptic and postsynaptic receptors with 5-MeODMT could be demonstrated for the descending 5-HT pathways using the sensitivity to noxious heat as a test for the function of these neurons (Berge & Hole 1979).

5-MeODMT in a volume of 25 µl was injected into the lateral ventricle of rats (N=8 for each dose). Low doses (1.6, 3.1, 6.3, 12.5 and 25 µg) produced hyperalgesia (maximum reduction of TF latency 24%). Fifty and 100 µg caused biphasic responses (hyperalgesia followed by analgesia) whereas 400 µg resulted in long-lasting (more than 90 min) analgesia (mean TF latencies 130–180% of preinjection scores).

Taken together with previously reported data indicating modulation of pain sensitivity via spinal 5-HT receptors (Berge & Hole 1979), these results suggest that also in the descending 5-HT system, 5-MeODMT in low doses preferentially stimulates presynaptic receptors leading to hyperalgesia due to reduced activity in descending fibers. Higher doses however cause analgesia probably by stimulating postsynaptic 5-HT receptors in the spinal cord.

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D 24

High affinity uptake of glutamate in terminals of the corticopontine pathway

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Evidence is accumulating that glutamate (Glu) may be the transmitter in several corticocortical and corticofugal pathways, and that high affinity uptake of [^{3}H]-L-Glu may be useful for tracing such pathways (e.g. Fonnum et al. 1979). The pontine nuclei receive a dense projection of fibres from the cerebral cortex. This excitatory projection (Sasaki et al 1970) is predominantly unilateral and appears to constitute by far the major input of these nuclei (see Brodal 1972).

The corticopontine fibres were severed in rats by transecting the cerebral peduncle just rostral of the substantia nigra by means of a tungsten wire extended about 1.5 mm at right angle through the tip of a cannula that was lowered stereotaxically across the ventrolateral surface of the peduncle. The animals were killed and the pontine nuclei were dissected under the microscope from fresh slices and homogenized in buffered 0.3 M sucrose to produce suspensions of nerve ending particles. High affinity uptake of [^{3}H]-L-Glu and [^{14}C]GABA (1 µM each) was measured by double labelling and protein content determined as described before (Storm-Mathisen & Woxen Opsahl 1978).

At 5 days after bilateral lesions the Glu uptake was reduced to 35 ± 3% (mean ± sem, 8 samples from 4 animals, P < 0.001) of the uptake in simultaneously assayed samples from sham operated controls. Survival for 7 or 12 days resulted in no further reduction. The value for GABA was 95 ± 9%. Similar results were obtained when [^{3}H]-D-aspartate was substituted for [^{3}H]-L-Glu. At 5 days after unilateral transections Glu uptake was 42 ± 6% ipsilateral and 77 ± 2% contralateral to the lesion.

These results show that afferents to the pontine nuclei descending through the cerebral peduncles possess Glu uptake capacity in their terminals. The fibres may be partly crossed. Terminals that take up GABA are predominantly of other origin.

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[1] Fellow of the Norwegian Agency for International Development (NORAD) from Mahidol University, Bangkok, Thailand

D 21

Effect of adrenalin on the tentacle reflex of Helix pomatia

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It is a common feature of contraction of many vital invertebrate organs that the speed and degree of contraction changes with repetitions of the initiating stimu lu (Horn & Hinde 1970; Kandel 1979) These movement changes possess a large number of properties that are qualitativ ly identical to learning phenomena in higher animals Further they are controlled by simple even monosynaptic neural networks in which the plastic element ha been localized to the synapse and in one case (Kandel 1979) de cribed a presynaptically caused depre sion (Chri toffersen & Juel 1979) As a supplement to the observation of a series of learning-like properties of the tentacle withdrawal of Helix pomatia (Chri toffersen in prep) one more feature of learning known from higher animals wa presently tested and found: sensitivity to adrenalin

Repeated electrical stimulation of an optic tentacle led to a fall of the frequency of associated withdrawal exten ion equences A control serie of timulations of the left tentacle for one hour was followed by injection of adrenalin (body concn approx 10⁻ M) Thereafter a one hour test serie w s performed on the right tentacle The reflex plasticity in the two tentacles is independent wherefore control and test series can be performed in the same animal

In 5 test experiments a significant elevation of the frequency curve was observed: before injection the area under the mean curve was 135 ± 8 (S D) timul ations as opposed to 185 ± 26 (S D) afte injection The tudents t-t st of the in crease of the area gave 0 02 p 0 05 The injection of pure Ringer in 4 control animals did not affect the curve significantly The elevation may be described as an increase of activity most likely due to enhancement of cardiac output (Dale 1973) Such activity change is generally known to take place and affect learning in vertebrates (Gold & Mc-Gaugh 1975)

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D 22

On the control of the central pattern generators for swimming in the isolated lamprey spinal cord in vitro

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The thin (200-300 μm) spinal cord of the lamprey (Ichthyomyzon unicuspis) can be maintained in vitro for several days in cooled Ringer If descending fibres in the cord are electrically sti mulated or if glutamic acid (0 6 1 2 mM) is applied to the bath coordinated motor activity can be recorded in the ventral roots (Cohen & Wallén 1978 Poon 1979) Bursts of motoneuronal discharge alternate between the two sides of each segment The ac tivity along the cord is coordinated with a cons tant phase lag between adjacent segments These are the characteristics of swimming in fish and accor dingly the spinal network can be said to generate fictive swimming (without movement) The motoneurones undergo periods of depolarisation and hyper polarisation (IPSPs) (Russell & Wallén 1979) The burst rate is markedly influenced by the pool temperature It could be changed between 3°C and 25°C (Typically kept at 7°C)

The central network appears to depend on post synaptic inhibitory mechanisms Application of the glycine antagonist strychnine influences the rhythmic activity When the dose is increased in several steps from 0 5 to 2 5 μM the frequency of alternation increases progressively At the highest do se the rhythm becomes very variable and bursts may be skipped Finally tonic activity occurs on both sides without reciprocal interactions The putative inhibitory transmitter glycine (1 mM in the bath) not only decreases the degree of efferent ac tivity but also lowers the rate of bursting and will finally abolish the rhythm GABA another putative inhibitory transmitter is less likely to be involved as its antagonist bicuculline (0 025-2μg/l) does not seem to influence the rhythmic activity to any significant degree

Mechano-receptors in or around the notochord can profoundly influence the central network controlling swimming One part of the notochord with the spinal cord was bent back forth while the other part was fixed in the dish This caused the rhythm to be modified and follow the imposed movement A movement towards the left side would induce left side acti ity and vice versa The receptors must be located in the notochord with adjacent connective tissue as muscle and skin have been removed Possible receptors in the spinal cord itself can be excluded as a transection of dorsal and ventral roots abolishes the dri ing effect These feedback effects are ana logous to those of the dogfish (G illner & Wallén 1977) and are similar to those of the cat hindlimb (Andersson et al 1978)

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D 23

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Taken together with previously reported data indicating modulation of pain sensitivity via spinal 5-HT receptors (Berge & Hole 1979) these results suggest that also in the descending 5-HT system, 5-MeODMT in low doses preferentially stimulates presynaptic receptors leading to hyperalgesia due to reduced activity in descending fibers. Higher doses however cause analgesia probably by stimulating postsynaptic 5-HT receptors in the spinal cord.

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D 24

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D 25

Post tetanic stiffnes in isolated frog muscle fibres

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Recent X-ray diffraction data (Yagi et al 1977) indic te that the return of the cross-bridges from the vicinity of the thin filam nts aft r a contraction occurs in two sta ges: A rapid return of the majority of the cross-bridges was followed by a slow return of the remaining 20% At a temperature of 4°C the low return stage started about 1 s after the end of the stimul tion nd even afte 5 s 10% of the cross-bridges had not yet returned

The purpose of the present experiments w s to e am ine whethe the return to the low resting value of the stiffness of the fibre followed a similar two-stage beha viour In th s way one would obtain n indic tion wheth er the slow return of the cross-bridges reflected a at tachment to th thin filaments The xperiments were carried out on 8 isolated semitendinosus m scle f bres (R temporaria) unde conditions similar to those of Yagi et al : sarcomere length 2 2 µm t mper ture 4°C; tetanic stimulation fo 1 s at a frequency of 20 Hz The stiffn ss w s measured by subjecting the fibre t a q ck stretch (ramp-and-hold) during the stimulation period and at variou times (p t 5s) fter the last stimulus In addition the tiffness of the re ting fibre was meas- ured s a ref rence

At th tim of the last stim l s (t 0) the stiffness of the f bre was about 200 time the re ting v lue The stiffness followed the decre se of the contractil force in an lmost proportional mann r At t 1 both f rce nd stiffness w only a f w (3-5) per cent of the v l ues me ured t the tim t 0 From about t 2 5 the contractil force w s less than 0 5% of the alu meas- ured at t 0 and the stiffness w s only bout 1 2 times the resting value corresponding to about half per cent of the value obtai ed at t 0 There w s no noticeable change i the stiffness during the interv l 5-5s After two min tes the stiffness had ret rned to its rest ing val e

The attachment of cros -bridges such as indicated by the stiffness of the muscle fibre in the post tetanic peri od thus did not follow the time course of the number of cross-bridges in the vici ity of the thin filaments It th refore appears as if the cros -bridges can be in the vicinity of the thin filaments without being attached to these X ray data for the ti e being can thus only g an upper limit fo how great a number of th cross- bridges re attached

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D 26

Training and the paradoxical infl ence of verbal encouragem t on mu cl fatigue

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18 healthy young male s bjects performed 150 repeated maximum voluntary muscle contractions (MVC) with one leg alone (right and left leg) and with both legs simultaneously before and after a training period of five weeks duration 5 ubjects performed the functions of controls (co trol group) 7 subjects performed 150 two -leg MVCs (two-leg gro p) and 6 s bjects performed 150 one leg MVCs (one leg group) three times a week In all series one con traction was performed every fifth econd Before and after the training period force and rectified smoothed electromyo graphy (rsEMG) were registered d ring verbal encouragement before and during each contraction During the training pe riod force was registered while the sub jects were working alone Results con cerning training effects on strength have been given (Rube et al 1979) Res lts on muscle fatigue are presented here

Before the training period force and rsEMG decreased in all subjects by 2 % and 33% respectively during the 150 re peated MVCs The ratio between force and rsEMG remained almost constant

At the beginning of the training peri od the initial force values were not sta tistically different from those of the initial val es obtained during the enco r ged contractions Force decreased by 5% and 10% for the two and the one leg gro ps respecti ely

Afte the t i i g pe iod the control group on an average showed a 20% decrease in force during the 150 MVCs The two-leg gro p showed an unchanged decrease in force during repeated one leg extensions but fatig e developed during repeated two leg exten ions was reduced by bo t 50% In the e leg gro p fatigue seen d ring repeated two leg extensions r main d prac tically constant whil one leg strength red ction was 36% smaller than prior to the training period

The es lts s gg t that the fatigue develop d during repeated super ised nd verbally encouraged MVCs is paradoxically i fl ced by the pres e of the in esti g tor Furthermore that tr ining r d c s thi type of fatigue i the pecific type of contractions pra tised

R f re e

RUBE N SECHER N H and LODBERG F 1979 Th eff ct of habituation and t i ing on two d ne leg ext nsi n t gth A t Phy i l Sc nd In pr s

Renal reabsorption f Na and K in Gallus rola f urinary precipitate

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Determinations f urate Na and K i whol urines and their supernatants in birds have produced [Na]/[urate] and [K]/[urate] molar ratios ignificantly greate than unity (McNabb McNabb and Hinton 1973; Braun 1978) I conjunction with measurements f glomerul filtration te and plasma electrolyte concentrations these findings indicate fr ctional reabsorption of filtered loads may be as low f exampl 0 63 f Na in tarlings in contrast to values above 0 99 f the single-phase urines f mammals

In the present tudi imila determinations were made directly on dissolved urinary precipitates of 6 hen Urine collected by catheters sewn ove ur teral orificia and tored t 39 5°C (body temperature) we centrifuged f 10 minute t 100 000 gravity Precipitates dried f 2 day over $CaCl_2$ were analyzed for [urate] by uricase digestion and f [Na] and [K] by flame spectrophotometry with tandards from 5 to 0 5 mEq/L

[Urate] i these solutions rang f om 8 2 to 5 4 mM/L [N] and [K] from er to 8 6 mEq/L Average [Na]/[urate] and [K]/[urate] molar ratios f 31 sample ar 0 111±0 016 and 0 100 0 013 (S E M) respectively; correction f Na and K in precipitate due to estimated trapped volume of supernatants produces till lower va ge The low molar ratios are in accord with y crystallogr phi data eporting that avian rinary precipitates ar primarily uri acid dihydr te with littl or no contribution f urate (Lonsdal and Suto 1971)

The molar ratios ar independent of urocrit (UT) the rati f dry precipi tate to whol urine weights UT ang from 0 8% in diureti birds fed commerci l di t to 18 6% in non-diur tic birds fed a high protein di t Likewise molar ratios showed no correlation with urinary [Na] (range 2 164 mEq/L) [K] (6 78 mEq/L) [ur te] (3-285 mM/L) in whol urine and supernatants or with urinary pH (4 5-6 9).

Our esults indicate no ignificant trapping binding f Na o K in precipi tated urates in hens urines F ctional reabsorption f N and K in the 20 sampl examined in these ix hens 0 982 0 003 and 0 794 0 014 respectively and imply mino rol for post renal recupe tion of these ion during loacal torage of urine.

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Skeletal muscl adaptation in man following prolonged exposur to high altitude

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In the acclimatization to chroni hypoxia many adaptations occur in organs and tissues some of these also occur in the skeletal muscl I has been suggested that denser capillary network and an elevated oxidative potential of the muscle accompany prolonged exposure to hypoxia (Cassin et al.1971 Tappan and Reynafarje 1957 Valdivia 1958) This concept has however ecently been at least partly challenged (Gillen and Banchero 1977)

A unique possibility to study the long term adaptation of lowlanders to high altitude occurred in connection with the building of an irrigation system in the Andes Nine men were studied befor (sea level) and after 6-44 weeks at altitude (3 450 m, bar pressure ≃ 510 mmHg) Muscle biopsies from the leg muscles were analysed or fibre composition and size capillarization and enzyme level The physical working capacity of each subject was also determined.

Fibr composition did not change at altitude The mean number of capillaries per fibre as well as the number of capillaries around each fibre type was unchanged. The sizes of the muscl ibre types wer smaller in 6 subjects Consequently with unchanged capillary to fibre ratio capillary density tended to increase and the area supplied by each capillary was reduced

Glycolyti enzymes did not change at al itude whereas citratesynthase was educed 9% (p<0 05) and 3-hydroxyacyl-CoA-dehydrogenase by 5% (p 0 05) The maximal oxygen uptake of the subjects averaged 3 12 l/min sea level and was onl 12% lower altitude

The present results are in contrast with the traditional concept but are in line with more ecen studies Thus shorter diffusion distances in the skeletal muscle can be part f the adaptive response of lowlanders to chronic hypoxia Thi is not brought about by proliferation of the capillaries but rather by reduction in the size of the muscle fibres. Further hypoxia per se does not appear to be stimulus for enhancement of the mi ochondrial enzyme level which therefore may not be part of the process of acclimatization to high altitude The high values reported earlier for skeletal muscle oxidative enzymes in highlanders might be explained by the use of subjects with high level of physical activity

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D 25

Post tetanic stiffness in isolated frog muscle fibres

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Recent X ray diffraction data (Yagi et al 1977) indicate that th return of the cross-bridges from the vicinity of the thin filaments after a contraction occurs in two st ges: A rapid return of the majority of the cross-bridges was followed by a slow return of the remaining 20% At a temperature of 4°C the slow return st ge st rted about 1 s aft r the nd of the stimulation and ev n aft r 5 s 10% of the cross-bridges had not yet returned

The purpose of the present *experiments was to e m ine* wh ther the return to the low resting value of the stiffnes of the fibre followed a similar two-stage beha viour In thi way one would obt i a indic tion wheth er the slow return of the cross-bridges reflected t tachment to the thin filame t The xperiment w re carried out on 8 isolated semitend nos m scl fibre (R temporaria) under condition simil r t those of Yagi et al sarcomere length 2 2 µm temperature 4°C tetanic stimulation for 1 s at freq ency of 20 Hz The stiffness was measured by subjecti g th fibre to q k stretch (ramp-and-hold) duri g the stim lation period nd t various times (up to 5 s) fter the last stimulus In add tion the stiffness of the resting fibre was meas ured as a ref renc

At the time of the last stim l s (t 0) the stiffness of the fibre was bout 200 time the resting value The stiffness followed the decre se of the contra tile forc in an almost proportional manner: At t 1 s both force and stiffne va only a few (3—5) per c t of the v l es me ured t the time t 0 From about t 2 5 s the contra til forc w s less tha 0 5% of the val e meas- ured at t 0 and the stiffness was only about 1 2 times the resting v l e c rrespondi g to about half a per cent of the value obtained at t 0 There w no noticeable change in the stiffness during the interval 2 5—5 s After two min tes the stiffness had returned to its rest ing value

The attachment of cross-bridges such s indicated by the stiffness of th muscle fibre in the post tetanic peri od th s did not follow the tim course of the number of cross-bridges in the vici ity of the thi filaments It therefore appears as if the cross-bridge can be in the vicinity of the thin filaments without being attached to *these X ray data for the time bei g ca* thus only gi e an upper limit for how great a number of the cross-bridges are attached

S pported by grant 511 15063 from the Danish Natural Science Research Council

R ference

YAGI N ITO M NAKAJIMA H IZUMI T & MATSUBARA I 1977 Science 197: 685-687

D 26

Traini g nd the parad io l infl nce of verb l encour gem t n muscle fatigu

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18 healthy young male subjects performed 150 repeated maximum voluntary muscle contractions (MVC) with one l g alone (right and left leg) and with both legs simultaneously before and after t aining period of five weeks duration 5 bjects performed the function of c ntrols (co trol group) 7 subjects performed 150 two leg MVCs (two leg gro p) and 6 bjects performed 150 one leg MVCs (one leg gro p) three times a week In ll series one co traction was performed every fifth second Before and after the training period force and rectified smoothed electromyo graphy (rsEMG) were registered during verbal encouragement before and du ing each contraction During the training pe riod force was registered while the s b jects were working alone Results con cerning training effects on strength h ve been given (Rube et al 1979) Results on muscle fatigue are presented here

Before the training period force and rsEMG decreased in all subjects by 25% and 33% respectively during the 150 re peated MVCs The ratio between force and rsEMG remained almost constant

At the beginning of the training peri d the initial force al es were not sta tistically different from those of the initial val es obtained d ring the encou raged contractions Force decreased by 5% a d 10% for the two and the one leg groups respectively

After the t ini g period the control g p verage showed a 20% decrea e in force during the 150 MVCs The two-leg group showed an nchanged decrease in fo e d ing repeated ne leg exte sions but fatigue de eloped during repe ted t o l g ext sions was reduced by about 50% I the one leg gr up fatigue e en d ring repe ted t o leg extensions remai ed prac ti lly t t hil one leg trength eductio was 36% s aller than prior t the trai ing period

The res lts ggest that the fatigue de el ped d ring epe ted sup r ised and verb lly e co g d MVCs is paradoxic lly i fl ced by the presence f th in sti g tor Furthermore that training r duc s thi typ of f tigue i the spe ific type of o t otio pr cti ed

R f nc

RUBE N SECHER N H nd LODBERG F 1979 The effect of habit ation a d trai ing o two and o l g xten ion trength A ta Physiol Sca d In press

D 31

Vascular characteristics in human renal adenocarcinoma.

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Departments of Surgery Radiotherapeutics Histology and Physiology University of Göteborg, Sweden.

In vivo and in vitro studies with microsphere tracer technique on induced DMBA rat mammary neoplasia have demonstrated that the tumors have relatively high resting blood flow high resistance during maximal vascular relaxation and strong response to noradrenaline (Weiss et al 1979 b) Pharmacoangiographic studies (i infusion f vasoactive drugs during angiography) on the contrary seem to support the hypothesis that tumor vessels of renal carcinoma are not abl to constrict (Ekelund et al 1976)

Against this background, surgically removed human kidneys with renal adenocarcinomas were subjected to haemodynamic investigation in an in vitro perfusion system where regional perfusion was studied by microsphere (∅ 15 µm) tracer technique Pressure/flow curves and dose/response curves for noradrenaline were obtained for the whol organ and regional vascular response was obtained t maximal vascular relaxation and at two different dosages of noradrenaline i at 3 points At the end of the experimen the preparation was perfused with formalin including suspension of barium sulphate to llow for specimen and section angiography illustrating vascular architecture and volume or visualization of perfusion inhomogeneities 2 mm thick sections of the whol organ including tumor were processed for autoradiography

The majority of the tumor tissue was poorly perfused From well-type scintillation analysis of well perfused tumo reas the peripheral resistance during maximal vascular laxation was found to be three times tha of renal cortex Gradually increasing infusion of noradrenaline caused rapid and marked increase of peripheral resistanc in tumor tissue which even seemed hyperreactive when compared to renal cortex

Angiography of the autoradiographed 2 mm sections illustrated discrepancy between ascular volume and perfusion as compared to renal cortex Tumor areas were heterogeneously supplied ith an i regular network f fine-caliber vessels Histopathological examinations were also undertaken trying to correlate morphology vascular architecture and perfusion

In summary the cross-sectional area f the precapillary resistance vessels of renal carcinoma is smaller than that f renal cortex further the tumor resistance vessels reacts strongly to noradrenaline and are probably hyperreactive compared to renal cortical resistance vess l The indication f tumor vascular hyporeactivity in pharmacoangiography can not be substantiated by this study but the discrepancy is probably due to the vastly diff ing levels in the vascular arborisation being studied

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D 32

ION CONDUCTANCE CHANGES IN HIPPOCAMPAL NEURONES

I MODEL

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When short current pulse is injected in neuron the membrane potential is changed and this change may be used to calculate the membrane resistance However the voltage response to hyperpolarizing current often deviate from the passive response expected from the membrane This nonlinear response was first described by Ito in the 1960 and later further investigated by Ito and Oshima in cat alf motorneurones They suggested that the non-linearities were produced by mechanism different from the mechanism causing delayed rectification and that more than one ion conductance might be involved

We have tested if the non-linearities may be due to changes in potassium conductance only by comparing recordings from in vitro guinea pig hippocampal slices and the outcome of the simulation of simpl computer model of the nerve membrane In the model the potassium conductance (G_K) was time and voltage dependent

$$\frac{dG_K}{dt} \quad G_K \quad \beta(G_{K\,max} \quad G_K)$$

$$k_1 \exp\{k_2(V \quad V)\}$$

$$\beta \quad k_3 \exp\{k_4(V \quad V)\}$$

where V is the resting membrane potential V the membrane potential and k_1 k_2 k_3 and k_4 chosen constants Thus the potassium conductance increases during depolarisation and decreases during hyperpolarization similar to the delayed rectification of the Hodgkin-Huxley model

The membrane potential was calculated during and after injection of current pulse by solving

$$\frac{dV}{dt} \quad \{I-G_K(V-E_K)-G_{Na}(V-E_{Na})-G_L(V-E_L)\}/C_m$$

by numerical recursive method on PDP 11/34 computer The G are ion conductances and the E are equilibrium potentials C the membrane capacitance

The membrane potential calculated in this model after current injections had time course very close to the time course recorded from CA1 in the hippocampal slice The constants k_{1-4} could be varied over wide range without changing the basic shape of the calculated potentials It is thus possible that the non-linearities are due to time and voltage dependent change in only potassium conductance Surprisingly the model also showed anomalous rectification in certain voltage range

R f renc s

ITO M & OSHIMA T 1965 J Phy i l
18 6 7-633

D 29

Uterine secret ry re ponse during the reproductive cycl of the guinea pig

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In previous report(1) evidence f choli nergic presumably sympathoti s cretory in nervation of the guinea pig endometrium we presented These xperiment were porf rmed on ut ri brought int artifici l secretory phase by combined tr tment with trogen and proge t rone Ut rine secretion has been calcula ted from amount f carbohydrat released from isolated verted guine -pig ut ri(1) Change in secretion in re ponse t fi ld timulation and/or admini tration f drugs were calculated per cent incre above re ting secretion l vel(1)

Since the endometrium unde goe change cording to the hormonal tat f the animal and a adrenergi myometrial nerve are sub j ted t change due t the serum levels of femal se hormone t dy f po ibl secretory re ponse during the normal trous cycl pregnancy well ft r treatment with ce f trogen and proge t rone we perfor med

In trous cy l both carbachol and fi ld timul tion increased secrotion cept during lat di trus In pregnancy cretory re pon se wa noted t implant tion time From mid pregnancy and onwards re ponse f increa ing magnitude were btained The secret ry respon se persi ted po t pa tum

Furthermore evidence f an adrenergi inhibi tory influence on the neurogenic cholinergic response wa obtained at mot true

Both carbachol and fi ld timulation increased carbohydrat secretion secretion by about 50% bove re ting se retion level in uteri f animal treated with both strogen and proge t rone In ut ri of animal receiving only strogentthe incre se wa only bout 20% N secretory re ponse were btained in ut ri f animal treated with proge terone only

From thi it could be suggested that both hormones are necessary f full holinergi secretory re ponse t fi ld timulation or to carbachol Furthermore it could be concluded that muscarinic timulation f the endomet rium results in mucus secretion during ll cruci l event f the reproductive cycl i t ovulation and impl ntation time during period f f tal growth and t delivery as well po t partum (when the para ovulat and normallly become f rtilised again) Furthermore cholinergi se retomot r innervation seems t be present t all these time

Ref rence

HAMMARSTRÖM M A SJÖSTRAND N O 1979 Acta Phy iol Scand 106 11 15 (1)

D 30

A comparison between freon and acetylene rebreathing for measuring cardiac output during rest and submaximal exercise

F BONDE PETERSEN P NORSK & Y SUZUKI

August Krogh Institute University of Copenhagen Denmark

The non-invasive rebreathing technique for measuring cardiac output (CO) has attracted renewn interest with the construction of mass spectrometers Kruhøffer (pers comm) uses freon 22 replacing acetylene (Grollmann 1929) as the soluble gas Triebwasser et al (1977) used the non soluble gas He in order to correct the fractions of the soluble gas for incomplete mixing and from a semi logarithmic plot of the corrected values CO was calculated

We measured CO in 10 subjects during rest and submaximal exercise using rebreathing of 2 2 5 l of 1% acetylene 3-4% freon 6 10% argon and 39-42% oxygen in nitrogen CO calculated from the two gasses correlated significantly (r=0 9923) but freon values were systematically lower This might be due to differences in the in vitro and the in vivo Bunsen solubility coefficients The results were comparable with those reported in literature Hyperventilation increased CO significantly at rest and low work rates This is attributed to a suction on the central veins demonstrated by an increase in central venous pressure At higher work loads there was a decrease in the rebreathing bag volume as indicated by an increase of the argon fraction due to CO_2 accumulation in the lung bag system which causes a decreased CO_2 excretion rate while O_2 is still taken up at the same rate This stresses the importance of using an inert gas as an indicator of incomplete mixing and volume changes because these two errors can be corrected for The increased heart rate during rebreathing was shown to be caused by the increased pCO_2

Conclusion Freon can replace acetylene as the soluble gas Spontaneous breathing is recommended An inert insoluble gas (He or Ar) must be used in order to give reliable results

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D 31

Vascular characteristics in human renal adenocarcinoma

E. TVEIT, R. [illegible], S. [illegible], L. WEISS

Departments of Surgery, Radiotherapeutics, Histology and Physiology, University of Göteborg, Sweden.

In vivo and in vitro studies with microsphere tracer technique on induced DMBA rat mammary neoplasia have demonstrated that the tumors have relatively high resting blood flow, high resistance during maximal vascular relaxation and strong response to noradrenaline (Weiss et al 1979 b). Pharmacoangiographic studies (i.e. infusion of vasoactive drugs during angiography) on the contrary seem to support the hypothesis that tumor vessels of renal carcinomas are not able to constrict (Ekelund et al 1976).

Against this background, surgically removed human kidneys with renal adenocarcinomas were subjected to haemodynamic investigation in an in vitro perfusion system where regional perfusion was studied by microsphere (ϕ 15 um) tracer technique. Pressure/flow curves and dose/response curves for noradrenaline were obtained for the whole organ and regional vascular response was obtained at maximal vascular dilatation and at two different dosages of noradrenaline, i.e. at 3 points. At the end of the experiment the preparation was perfused with formalin including a suspension of barium sulphate to allow for specimen and section angiography illustrating vascular architecture and volume. For visualization of perfusion inhomogeneities, thick sections of the whole organ including tumor were processed for autoradiography.

The majority of the tumor tissue was poorly perfused. From well type scintillation analysis of well perfused tumor areas the peripheral resistance during maximal vascular relaxation was found to be three times that of renal cortex. Gradually increasing infusion of noradrenaline caused a rapid and marked increase of peripheral resistance in tumor tissue, which even seemed hyperreactive when compared to renal cortex.

Angiography of the autoradiographed 2 mm sections illustrated a discrepancy between vascular volume and perfusion as compared to renal cortex. Tumor areas were heterogeneously supplied with an irregular network of fine-caliber vessels. Histopathological examinations were also undertaken trying to correlate morphology, vascular architecture and perfusion.

In summary, the cross-sectional area of the precapillary resistance vessels of renal carcinoma is smaller than that of renal cortex; further, the tumor resistance vessels reacts strongly to noradrenaline and are probably hyperreactive compared to renal cortical resistance vessels. The indication of tumor vascular hyporeactivity in pharmacoangiography can not be substantiated by this study, but the discrepancy is probably due to the vastly differing levels in the vascular arborization being studied.

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D 32

ION CONDUCTANCE CHANGES IN HIPPOCAMPAL NEURONES IN VITRO

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When a short current pulse is injected in a neuron the membrane potential is changed and this change may be used to calculate the membrane resistance. However the voltage response to hyperpolarizing current often deviate from the passive response expected from the membrane. This non-linear response was first described by Ito in the 1950s and later further investigated by Ito and Oshima in cat alfa motorneurones. They suggested that the non-linearities were produced by a mechanism different from the mechanism causing delayed rectification and that more than one ion conductance might be involved.

We have tested if the non-linearities may be due to changes in potassium conductance only by comparing recordings from in vitro guinea pig hippocampal slices and the outcome of the simulation of a simple computer model of the nerve membrane. In the model the potassium conductance (G_K) was time and voltage dependent:

$$\frac{dG_K}{dt} = \alpha G_K - \beta(G_{K\,max} - G_K)$$

$$\alpha = k_1 \exp\{k_2(V - V_r)\}$$

$$\beta = k_3 \exp\{k_4(V - V_r)\}$$

where V_r is the resting membrane potential, V the membrane potential and k_1, k_2, k_3 and k_4 chosen constants. Thus the potassium conductance increases during depolarization and decreases during hyperpolarization, similar to the delayed rectification of the Hodgkin-Huxley model.

The membrane potential was calculated during and after injection of a current pulse by solving

$$\frac{dV}{dt} = \{I - G_K(V - E_K) - G_{Na}(V - E_{Na}) - G_L(V - E_L)\}/C$$

by a numerical recursive method on a PDP 11/34 computer. The G are ion conductances and the E are equilibrium potentials, C the membrane capacitance.

The membrane potential calculated in this model after current injections had a time course very close to the time course recorded from CA1 in the hippocampal slice. The constants k_{1-4} could be varied over a wide range without changing the basic shape of the calculated potentials. It is thus possible that the non-linearities are due to a time and voltage dependent change in only potassium conductance. Surprisingly the model also showed anomalous rectification in a certain voltage range.

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D 29

Ut rine cretory re ponses during the reproductive cycl of the guinea pig

M HAMMARSTRÖM

Department f Physiology I Karolin ka Institut t Stockholm Sweden

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D 30

A comparison between freon and acetylene rebreathing for measuring cardiac output du ing rest and submaximal exercise

F BONDE PETERSEN P NORSK & Y SUZUKI

August Krogh Institute University of Copenhagen Denmark

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WEISS, L, HULTBORN, R, TVEIT E 1979 Microvasc Res 17 119

WEISS L, TVEIT, HULTBORN R 1979 Acta Physiol Scand, In press

D 32

SLOW CONDUCTANCE CHANGES IN HIPPOCAMPAL NEURONES IN VITRO

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When a short current pulse is injected in a neuron the membrane potential is changed and this change may be used to calculate the membrane resistance. However the voltage response to hyperpolarising current often deviate from the passive response expected from the membrane. This nonlinear response was first described by Ito in the toad and later further investigated by Ito and Oshima in cat alf motorneurones. They suggested that the non linearities were produced by a mechanism different from the mechanism causing delayed rectification and that more than one ion conductance might be involved.

We have tested if the non-linearities may be due to changes in potassium conductance only by comparing recordings from in vitro guinea pig hippocampal slices and the outcome of the simulation of a simple computer model of the nerve membrane. In the model the potassium conductance (G_K) was time and voltage dependent

$$\frac{dG_K}{dt} = B(G_{K\,max} - G_K)$$

$$k_1 \exp(k_2(V - V))$$

$$B = k_3 \exp(k_4(V - V))$$

where V is the resting membrane potential, V the membrane potential and k_1, k_2, k_3 and k_4 chosen constants. Thus the potassium conductance increases during depolarisation and decreases during hyperpolarization, similar to the delayed rectification of the Hodgkin-Huxley model.

The membrane potential was calculated during and after injection of a current pulse by solving

$$\frac{dV}{dt} = (I - G_K(V - E_K) - G_{Na}(V - E_{Na}) - G_L(V - E_L)) / C$$

by a numerical recursive method on a PDP 11/34 computer. The G are ion conductances and the E are equilibrium potentials, C the membrane capacitance.

The membrane potential calculated in this model after current injections had a time course very close to the time course recorded from CA1 in the hippocampal slice. The constants k_{1-4} could be varied over a wide range without changing the basic shape of the calculated potentials. It is thus possible that the non-linearities are due to a time and voltage dependent change in only potassium conductance. Surprisingly the model also showed anomalous rectification in a certain voltage range.

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D 33

Cardiac receptors and sympathetic nerve traffic in the spontaneously hypertensive rat (SHR)

S E RICKSTEN T YAO B LJUNG & P THOREN

Department of Physiology University of Göteborg Sweden

Cardiac mechanoreceptors mainly located in the left atrium in the rat are reset in SHR The threshold in left atrial pressure (LAP) for activating these receptors is twice as high in SHR compared to the normotensive rat (NR) (Thorén et al 1979) The difference in LAP threshold between SHR and NR for reflex inhibition of the renal sympathetic outflow from left atrial receptors was similar to the threshold difference for the afferent traffic from the receptors (Ricksten et al 1979) To explore whether structural changes of left atrium could explain the resetting in SHR the dynamic distensibility of the nonbeating isolated left atrium was studied in SHR and NR determining relative changes of left atrial volume upon a rapid LAP increase from 2 5 to 12 5 mm Hg The dynamic distensibility was 25% lower in SHR ($p < 0\ 01$), which to some extent can explain cardiac reflex resetting. One also has to assume a primary receptor change per se in SHR In awake rats a blood volume expansion inhibits the splanchnic nerve traffic significantly more in SHR compared to NR (Ricksten et al 1980) This was due to an augmented low pressure receptor response in SHR in spite of the receptor resetting probably due to a decreased distensibility of the capacitance vessels Therefore LAP was measured in awake SHR and NR during blood volume expansion From preliminary experiments a 20% volume load increased LAP significantly more in SHR compared to NR (4 4 vs 2 8) due to a greater centralisation of the volume load because of stiffer capacitance vessels in SHR compared to NR

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D 34

Hypothermia and bile production

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It has previously been demonstrated that total bile production is sensitive to small change in body temperature (Krarup & Larsen 1972) The purpose of the present experiments was to examine the effect of temperature on the different fractions of bile production i e canalicular bile acid-dependent and independent bile production and net ductular fluid transport

The experiments were performed on fasting chloralose-anestheti ed cats Bile was collected from a catheter placed in the choledochus Bile acids were infused at a rate of 0 2 µmol kg^{-1} min^{-1} Canalicular bile flow was estimated from the biliary clearance of ^{14}C-erythritol In the control period body temperature w s kept close to 38 °C and the temperature was then lowered to 36 °C by external cooling

In 5 experiments cooling caused a significant fall in total bile flow from 13 4 ± 1 8 to 8 9 ± 1 1 µl kg^{-1} min^{-1} together with a parallel decrease in canalicular bile flow from 18 6 ± 1 8 to 13 5 ± 1 3 µl kg^{-1} min^{-1} without affecting the *biliary* excretion rate of bile acids and the net ductular fluid absorption In 5 experiments the net ductular fluid absorption was changed to net fluid secretion by secretin 2 U kg^{-1} h^{-1} Again cooling decreased significantly both total bile fl from 22 6 ± 2 3 to 17 5 ± 1 6 µl kg^{-1} min^{-1} and canalicular bil production f 19 6 ± 2 7 to 14 8 ± 2 0 µl kg^{-1} min^{-1} without affecting the *biliary excretion* rate of bile acids and the secreti indu net ductular fluid secretion

Cooling thus decreases the bile acid in depend nt fraction of canalicular bil production apparently without influencing other proces es involved in bile production

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KRARUP N & LARSEN J A 1972 Acta Physiol *S and 84*:396 407

me cell aggregation in the renal medull consti ... as important facto in acute enal failure?

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Department f Physi logy and Medical Biophysics Biomedical Center Uppsala, Sweden

cute renal failure was induced by 45 min lamping f the renal artery (warm ischemia). A typical p t ... of response appeared with f i ly intac to... al renal blood flow grea ly dep essed glomerular filtration to 5 % of the normal isostenuria ... an impaired potassium secretion The reduced filtration was found to be due an increased ... tubular pressure probably secondary to mechanical obstruction located to the loop f Henl ... the medullary collecting duct

Although the total blood flow remained largely unaltered the medullary blood flow was depressed about 10 % f the normal, as investigated with ... rubidium extraction technique

The red cell content f the renal medulla as investigated from the equilibrium concentration f ... -Cr-labelled red cells was about 9 6 ± 0 43 % under normal conditions increasing to 22 1 ± 1 9 % (mean ± SEM) in conditions f acut renal failure The surplus red cell seems lso to be tagnant ... the vasa recta system

From these studies he sequence f events providing ... basis for acute renal failure is sugges ed to ...

The primary ischemia evokes functional alterations ... the capillary endothelium with subsequent formation of red cell aggregat in the vas recta system These aggregates will then constitut mechanical hindrance to the blood perfusion The ischemia will then proceed even after reci lation. Electron microscopical examination how that the original ischemia does not produce any cellular hange charact rized as irreversibl (Harvig-79) The continuing ischemia af er recirculation turn these cell into an irrever ibl tate with intracellular oedema and eventually necrosis wi h rejection f cell material into he tubular lumen in he loop of Henl and the medullary collecting ducts The cell debri will hen form obstructions for the urinary passage. As the filt ation continues ubular fluid will be accumulated with subsequent dilatation f the tubules and increased hydrostatic pressure up t level where the filtration ceases. Micropuncture experiment have verified hese conclusions

The depressed medullary blood flow will lso cause functional impairment f the renal medulla with subsequent los f the urine concentration ability and the ability to secrete potas ium

I hould be pointed out howeve tha some vasa recta escapes the deposition f ed cell aggregates The surrounding tubular tructures will then remain intact These nephrons will be the ones re ponsibl for he small filtration l f The number f intact nephron units det rmines whether the renal failure is polyuria or liguri but ther are no othe principle differences between hese two condi ions

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D 36

Correlation between bi logi al and i ologic l ... of rculatin growth hormone i normal ...

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There are conflicting report regarding the relation between bio- and immuno-assayabl plasma growth hormone (GH) activi ies Ellis and Grindeland (1978) have reported hat there appear to be much more GH-like activity in plasma of normal humans and rat as measured by the rat tibia assay than can be accounted for by radio-immunoassay (RIA) Stewart et al (1977) found good correlation between bio- and immuno-assayabl GH activi ... in plasma of rat bearing the GH secreting tumor (MtT/W15) both when bio-assayabl GH activity was det rmined in vitro (uptake f 3-O-methyl glucose) and in vivo (incorporation f thymidine into rat costal cartilage) The relation between bio- and immuno-assayable GH activities in pl sma f normal animal with an episodi secretory pattern f GH determined by RIA, has not been studied ... now

Freel behaving 30-day ld mal rats were decapitat d, he blood wa collected and plasma levels of GH were determined by highly specifi RIA (Isaksson et l 1978) Plasma levels of GH ranging between 0 and 800 ng/ml wa found Plasma f rat ... ining level lower than 8 ng/ml were pooled (pool) and plasma of rat containing more han 100 ng/ml were pooled (pool B) The two separat plasma pools were diluted with Krebs bicarbonate buffer (KEB) 1 2 Epididymal adipose tissue f hypophysectomized rat were preincubated with I) plasma from hypophysectomized rat diluted wi h KEB 1 2 II) bGH (1 g/ml) III) pool A and IV) pool B f 180 min Subsequently the effect f second addition f bGH (5 μg/ml) in vitro on the production f CO_2 from radioactive glucose was determined during the fourth hour of incubation The second addi ion f bGH increased CO_2 production in fat pad preincubated with (I) by 43 % (15 p 0 05) with (II) by 5 % (n 8 NS) wi h (III) by 46 % (15 p 0 01) and with (IV) by 7 % (10 NS)

The plasma of rat wi h high concentrations f irculating GH induced refractoriness to second exposure of GH highly specifi response in he ime course of action of GH. The present study suggests good correlation between bio- and immunoassayable GH activi ies

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D 37

The influence of potassium-induced neuronal nor adrenaline release on the potassium sensitivity of mesenteric resistance vessels in young and adult spontaneously hypertensive rats

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Potassium activates vascular smooth muscle by depolarizing *the plasma membrane and thus increasing* the permeability of potential controlled Ca channels (Bolton 1979) Recent evidence suggests that the conflicting evidence concerning the K sensitivity of the vasculature from spontaneously hypertensive rats (SHRs) may in part be due to the effects of neuronal noradrenaline (NA) release induced by K stimulation (Vanhoutte 1978) We have therefore investigated the K sensitivity of mesentric resistance vessels (i d 150-200 µm) from young (4-wk BP=52±3(SE) mmHg n=10) and adult (4-mo BP 154±5 mmHg n=9) SHRs Control vessels were taken from normotensive Wistar Kyoto (WKY) rats (4-wk *BP=53±3 mmHg n 10 4-mo BP=99±4 mmHg* n=9)

Vessel segments were mounted on a myograph (Mulvany and Halpern 1977) and their K-characteristics determined in cumulative K-dose response experiments before and after treatment to eliminate the effect of K induced neuronal noradrenaline release Treatment consisted of destroying the nerve terminals in vitro with 6-hydroxydopamine (Apprigliano & Hermsmeyer *1976*) or of blocking the α-receptors with phentolamine (1 µM)

The treatment (a) reduced the maximum K response (with 125 mM K^+) substantially but (b) did not affect the K responses at K-concentrations *below* *about 50 mM Thus the treatment* did not affect threshold K sensitivity but reduced the K-ED_{50} concentrations The reduction in the maximum K responses caused by treatment was similar in SHR and vessels *but greater in adult vessels (53±2%)* in the young vessels (36±3%) Before treatment adult SHR vessels were more (P<0 01)sensitive (K ED_{50}=48±2mM) than the WKY vessels (K ED =6 mM) but there was no difference in the K sensitivities of the young vessels (K ED_{50}=66±3 mM(SHR) 63±3 mM (WKY)) After treatment there was no difference in the sensitivities of the adult v (K ED_{50} 39±1 mM (SHR) 37±1mM (WKY)), but the y SHR vessels were less (P< 01) sensitive (K ED 44±1 mM)

The results therefore suggest that K induced neuronal NA release has a large influence on th *K responses of mesenteric resistance vessels* T the increased K sensitivity of untreated adult vessels does not seem to be due to an increased sensitivity of the smooth muscle within them H ever the decreased K sensitivity of the young t ted SHR vessels suggests because it precedes t development of elevated blood pressure that an initially decreased K sensitivity in the vascul could be a factor which is involved in the etio of hypertension in the SHP

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D 38

Facilit tory interaction between reflex pathways from muscle afferent of different diameter

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A wid convergence of diff rent aff rent onto common interneurones in reflex pathways from group I and II muscle afferents t α-motoneurone has been shown by testing spati l facilitation (Pedina & Hultborn 1972 Lundberg Malmgren & Schomburg 1077 1978) The technique of lective chemical acti tion of group III and IV muscl afferent (Mense 1977) enabl the investigation of their infl ence upon othe reflex pathways without ontaminating effects from low threshold aff rent

I high spinal cat motoneurones t hindlimb flexors (PBSt Per) and extensors (ABdm, GS Tib) were int ac llularly recorded and the effect of group III and IV muscl afferent activated by i tra arterial injection of potas ium chloride or bradykinin into the GS muscl upon reflex re ponses evoked by electrical timulation of group I II and III afferent from diff rent muscles was tested The reflex responses were llied to the different aff rent fibre groups according to thei threshold *stimulus* trength

EPSP evoked by group Ib II or III mu cl af ferents were almost gener lly facilitated by the afferent inflow from the chemic lly acti ted group III and IV muscle afferents Thereby it was irrel evant from which muscle the group I III afferent were originating and if they projected ont a flex or or extensor motoneurone except that Ib EPSPs in extensor motoneurones were not observed The facil it tion often outlasted the ynaptic membrane fects evoked by the group III and IV afferents Kniffki Schomburg & Steffens 1979) Thi fact gether with the obs rvation that monosynaptic I EPSP were ot enhanced sugge t that the ffect are not due to changes of the motoneuron l mem brane Group Ib nd II IPSPs principally ould be facilitated but the result were more variab and here often an influenc of the changed moto neuronal membrane potential could ot be exclud

The results indicate that there is an xtensi convergence from group III and IV muscl affere ont common int rneurone in reflex pathways fr group Ib II and III muscl aff rent Since re eptors of group III and IV muscl aff rents ar ot only nociceptive but l o can be activated muscle tretch and contraction (Kniffki Mense Schmidt 1978) it appears that the afferent of ferent mu le receptors may co-operate i the control of normal movements

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D 39

n relationship between insuli l val in- h Binding and response to insulin in lated f t cells from normal, ventromedial pothalamic lesioned and diabeti ts

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Insulin binding and ^{14}C labelled glucose in-corporation into f tty acids war tudied in isolated f t cells from ventromedial hy-pothalamic (VMH) lesioned ts (Hustvedt & Løvø 1972) weighing more than 350 g in weight matched controls and in ts weigh-ing about 240 g with or without streptozoto-cin induced diabetes

The insulin binding was ignificantly in-creased in adipocytes from VMH lesioned (P <0 01) and diabetic (P<0 02) ts compared to control ats (Table I) The increased binding in diabeti rats was associated with lower plasma insulin concentration I contrast the insulin level in VMH lesioned rats tended to be elevated indicating that factors othe than plasma insulin regul te the receptor concentration

The glucose incorporati into f tty acids was considerably elev ted in the VMH lesioned rats as compa ed with weight mat ched controls (P<0 01) Streptozotocin in-duced insulin depl tion lowe ed the glucose incorporation i to f tty cids (P 0 01 Tab-le I) even though recepto binding was in creased The results sugge t that the re to-tion of insulin-induced f tty id synthe-sis in VMH lesioned rats i not caused by the increased binding to the receptors

Tabl I Insulin binding and insulin-stimu-lated f tty acid synthesi

Binding Isolated adipocytes were incubated 37°C for 50 min with 25 pM ^{125}I-monoiodoinsulin (Gliemann & Sonne 1978) Results are expressed as specific binding (total binding minus binding in presence f 1 μM unlabelled insulin) as cpm per cell/cpm per 1 medium 10^{-11} i.e fractional binding physio-logical concentration. Fatty acid synthesis Isolated adipocytes were incubated in the presence of maxi mal concentration f insulin and 4 7 mM (U-^{14}C)gluco-se for 2 hours 37°C. The lipids were extracted hydrolised and the ^{14}C-activity was measured in the fatty acids Results are expressed as nmol glucose converted/10^5 cells/2 hour Median and range

Group	240 g n=10	Diabeti n=10	VMH n=12	350 g n=7
Binding	1 5 0 8-1 9	2 6 0 7 3 4	2 9 1 7-4 0	1 6 1 0 2 7
F tty ids	10 5-99	1 0-5	84 10-229	9 0-20

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D 40

Role of 5-hydroxytryptamine and vasoactive intestinal polypeptide in the pathogenesis of cholera secretion

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Recently it was showed from this laboratory that blocking f intramural nerves markedly inhibited the intestinal secretion induced by cholera enterotoxin (Cassuto et al 1979) Based on these result and on observa ions in other laboratories the f llowing hypo-thesis was proposed f the nervously mediated choleraic secreti as N rvous reflex( ) are activated via the release f 5-hydroxytrypta-mine (5-HT) from the ent rochromaffin (EC) cells. The 5-HT timulate dend ites located closely adjacent to the EC lls One f the transmitters released t the fferent end f the reflex arc( ) in the rypts and/or in the villi i vasoactive intestinal polypeptid (VIP) The f llowing experiment are perf rmed to test thi hypothesi 1 Rats exhibiting choleraic secretion were ither med tachyphylactic against serotonin or 5-HT receptor blocking agent (methysergide hlorpromazine) were given intravenously All thes experimental manipulations inhibited the holeraic secretion and in most cases an bsorption was induced The mean ffect was decreased secretory response of about 60% f the t tal trans-port change caused by the cholera terotoxi In accordance with these results histochemical observations the EC lls in cats show marked reducti f 5-HT ontent after th in ducti f choleraic secreti (Nilsso et al this supplement) 2 In cat experiment the release f VIP into the blood from the gut was d termined in control animal and in animals exposed to holera toxin During 5 h be r-vation period there was co tinuous decreas in the VIP release in the co trol animal while the VIP releas increased steadily from intestine exposed t the holera toxin. Thi release could be inhibited by tetrod toxin given i or lidocain administered into the gut lumen The present xpe iments are thus in f ll agreement with the hypothe i out lined here

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D 41

The ffect of high f t di t on adipos ti ue lipoprot i lipase activity i t

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Human obesity can be di ided in two main cat gor-i Usually childhood besity is hyperplastic while be ity riginating l t r in lif is hyper-trophi Hyperplasti besity can be studi d with Zucke at which are genetically bese (Gruen & l. 1978) The best approach fo hype trophi type i probably through di tary manipul tions

The lipoprot i lipas (LPL) enzyme cat lyse hydrolysi f irculating t iglycerides releasing free f tty acid to be transported t adipocyte Thus this enzyme i of importance in the develop-ment f obesity (Gruen & l 1978; Hi tanen & Greenwood 1977) In the present study r t were f d high f t di ts and the ff t f these di ts on the adipos tissue LPL activity were tudied

Rats were fed high f t di t (24 5 J/g) high f t diet upplemented with ext prot i (23 9 J/g). *Di tary protein content was 18 % in normal high fat* di t and 26 5 % in prot in-enriched high fat di t The feeding was t t d at weaning and if first consi ted only f high f t di t whil aft 3 weeks r t were divided to two group havi g both high f t di t but diff rent prot in upplementation Feeding thes xpe imental di t was f rthe con-tinued f 6 weeks Rats were killed and epididymal f t pad were removed The f t pads were homoge-nized in 0 25 M ucrose-1 mM EDTA and postmito-chondrial supernatant was prepared f enzyme assays The lipoprot i lipase (LPL) was determi as described by Hi tanen & Greenwood (1978) The adipos tissue DNA content (Greenwood & Hirsch 19) and prot in content i the postmitochondrial supe natant (Lowry & l 1951) were measured

The weight gai was significantly faster in ra having high fat di t suppleme d with prot in F pad weight and rel tive f t pad weights did not diff r between groups but if compared to rats fed diet with normal f t content the rel tive fat pad weights were higher in thos rats fed high fat di t Th DNA content was significantly higher i those fat pads taken from rat having high fat di than i those having high fat di t supplemented with prot i The LPL activity wa ignificantly higher in the high f t group than in the high fat group with prot i supplement when expressed per wet weight (13+2 vs 5 4 1 0 µmol FFA/h) Also protein basi the LPL activity was higher in the high f t group without prot in supplementation However when the ctivity is expressed per mg of DNA there was no significant difference in the tiviti s

The re ults ugge t that in rats having high f di t the LPL activity increase precede the en-l rgement f adipocyt and coincide with the ce prolife tion Grant: Y Jahn son Foundation (Finl

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D 42

Regul tion f glycog n phosphoryl se nd gly-coge synthetase during xercise in ma

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Thi tudy was unde tak to determine if the increased rat f glycoge breakdown d ing exercise wa due to t ansf rmation f phospho-rylase b t and t inve tigat the l f

AMP i thi proces Seve ubjects work d to exhaustion on an rgomet ycl (4 8 min) Muscl ampl were tak by the ne dl biop y technique bif re and afte xercise nd were analysed f cAMP glycogen pho phoryl se and glycogen ynthete I and D lactat and hexo-phosphates The muscl conte t f cAMP incre-sed bout 100% during xercise and the active f rm of glycoge syntheta decreased from about 40 % f the total acti ity t re t to bout 10 % fter xercise An unexpect d finding howeve that pho phoryl se decreased f om about 10 % f the t tal activity t re t t about 4 % afte exercise Blockade f th ceptors bef re xerci with prop anol l in-hibit d th increa i cAMP nd the glycoge synthetase transf rmation during xercise Pho phoryl se was after xe rcise with -blockade further de re sed (bout 1 % f the total phosphorylase activity) Accumul tion f lactat and glycos 6-phosphate i mus l were however ft excercise with -blockade (84 and 2 2 umol/g dry wt re pectively) s compared to Excercise without blockade (101 and 8 7 umol/g dry wt spectively)

.ncreases in vascula permeability induced by ygen free radical

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y. Exp Med, Pharmacia AB Uppsala

ygen toxicity i suggested to be manif ted by production of reactive intermedia es during biological reduction of oxygen to water Al ough most oxygen i reduced direc ly to water th the addition of four lectrons by cytochrome idase univalent reduction occur resul ing in peroxide radical (O_2^-) hydrogen peroxide (H_2O_2) d hydroxyl radical (OH) Many oxidative pro- sses of biological importance have been shown produce O_2^- In vitro experiment illustrat at the damaging ffects of O_2^- appears to depend the simultaneous presence f H_2O_2 which can formed by the spontaneous dismuta ion f O_2^- reason for thi seems to be tha O_2^- and H_2O_2 react to form OH which i one f the most active oxidizing species known

Enzymatic defense mechanisms against these reac- ve oxygen intermediates have evolved superox- de dismutase (SOD), talase (CAT) and gluta- thione peroxidase in addi ion to α-tocopherol d ascorbate These protective mechanisms are stly intracell lar leaving the extracell lar ace more susceptibl to oxygen adical attack lymorphonuclear leukocytes whi h participat in flammatory reactions can be activated by va- ety of timuli to grea ly incre se heir oxygen nsumption. This "respiratory burst is associa- d with the production f O_2^- some of which is leased into the extrac ll lar space where i may give ise to more reactive oxidizing specie such as OH

We have tudied the ffec f enzyma ically pro- duced oxygen derived free radical on ascular per- meability using the hams er cheek pouch model FITC-dextran (Mw 150000) was injected i v as permeability trace and O_2^- and H_2O_2 gene tion accompli hed by the opical applic tion f xan- thine oxidase and hypoxanthine Thi caused ig- nifican increase in numbe of leak per cm The simultaneous applica ion of ither SOD CAT di methyl sulfoxide or L-methionine ignificantly re- duced the leakage suggesting OH the main da- maging specie

These sul suggest that oxygen derived free r- dical may cause an increase in vascular permeabi lity and tha degree of the increase in permea- bility seen in inflamma ion might be related to oxygen adicals produced by the acti ity f inva- ding leukocytes

D 41

The ff ct f high fat di t on adipose tissue lipoprot in lipase a tivity in rat

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Human obesity can be divided in two main cat gories Usually childhood be ity is hyp rplastic while obesity riginating late in life is hypertrophi Hyperplasti obe ity can be studied with Zucker rat which are genetically obese (Gruen & al 1978) The best pproach f r hypertrophic type i probably through di tary manipulations

The lipoprot in lipas (LPL) enzyme catalyse hydrolysis f circulating triglyceride reles ing free fatty acids t be transported t adipocyt Thus thi enzyme i f importance in the development f obesity (Gruen & l 1978; Hi tanen & Greenwood 1977) In the present study rats were fed high f t di ts and the ffe t f these di t on the adipose tissue LPL activity were studied

Rat were fed high f t di t (24 5 J/g) r high f t di t supplemented with extr prot in (23 9 J/g). Di tary protein content was 18 % in normal high fat di t and 26 5 % i prot in-en iched high f t di t The feeding was tart d t weaning and if first consisted only f high f t diet whil aft 3 weeks rat were divided t two group having both high fat di t but diff rent prot in upplementation Feeding thes experiment l di t wa furth continued f r 6 weeks Rat were killed and epididymal f t pads were removed The f t pads were homogeni ed in 0 25 M sucrose-1 mM EDTA and postmitochondri l upernatant was prep red f enzyme assays The lipoprot in lipase (LPL) was as described by Hi tanen & Greenwood (1978) The adipos tissue DNA content (Greenwood & Hirsch 1[illegible] and prot in content in the postmitochondri l sup natant (Lowry & al 1951) were measured

The weight g in was significantly faster in r having high fat diet upplemented with protein pad weight and relative fat pad weights did not diff r between groups but if compared to r ts f[illegible] di t with normal fat content the rel tive fat p[illegible] weights were highe in those rats fed high fat di ts Th DNA content was significantly higher those fat pads taken from rats having high fat [illegible] than in those having high f t di t supplemented with protei The LPL activity was significantly higher in the high fat group than in the high [illegible] group with prot in supplement when expressed per wet weight (13+2 vs 5 4 1 0 µmol FFA/h) Also prot in basis the LPL ctivity was higher in the high f t group without prot in supplement tion. However when the activity i xpressed pe mg [illegible] DNA there was no ignificant diff rence in the activiti s

The re ult suggest that in r t having high di t the LPL ctivity increase precede the en largement of adipocytes and coincides with the c prolife tion G ant: Y Jahnsson Foundation (Finl

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D 42

Regul tion f glycog n phosphoryl se and glycoge synthet se du ing xercise in ma

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This tudy was undertak to det rmine if the incre sed te f glycogen breakdown du ing exercise was due to t ansf rmation f phosphoryl se b t and t inve tig t the rol f AMP i thi proce Seve subjects work d t exhaustion on an rgomet r ycl (4-8 min) Muscl sampl s were tak n by the ne dl biopsy technique bif re and ft r xercise nd were analysed f r cAMP gly oge phosphoryl se and b glycoge synthetas I and D l t t nd hexo se phosphat The mus l conte t f cAMP incre sed bout 100% during xercise and the active f rm of glycogen ynthetas decre sed from about 40 % of the total activity t re t to about 20 % after xercise An unexpect d finding wa however that phosphorylase de sed from bout 10 % of the t tal activity t re t t about 4 % afte exercise Blockade f the re ept rs bef re exerci with propranol l inhibit d the increa i cAMP and the glycoge ynthetase transf rmation during xercise Phosphorylase a was afte xc rcise with -block de further decre sed (bout 1 % f the t tal phosphoryl se activity) Accumul tion f la tat and glycos 6-phosphat i muscl were lower fte exe rcise with -blockade (84 and 2 2 umol/g dry wt re pectively) as compa ed to exercise without blockade (101 nd 8 7 umol/g dry wt respectively)